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UNIVERSITY OF ALBERTA

Dopamine as an inhibitory neurotransmitter candidate in the hydrozoan *Polyorchis penicillatus*.

by

JUN-MO CHUNG

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

SPRING 1991

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DEDICATION

To GOD,

To my parents,

To my brother and sister,

To Jin-Hee, Gemma, and my beloved wife Yeon-Sook

Who have consistently supported scientific inquiry

ABSTRACT

The identity of neurotransmitters at cnidarian chemical synapses remains a mystery, despite considerable attention. High performance liquid chromatography of alumina extracts of several tissues in *Polyorchis penicillatus,* one of the hydromedusae, showed the presence of dopamine and unidentified catechol-like compounds. Dopamine was found in the highest concentrations in nerve-rich tissue, at intermediate concentrations in endoderm-rich tissue, and at the lowest concentrations in the mesoglea. The dopamine was not likely to be from exogenous sources. The content of dopamine in nerve-rich tissue depended on the Ca^{2+} content of the dissecting media. The presence of dopamine in nerve-rich tissue was confirmed using gas chromatography-mass spectrometry with negative ion chemical ionization. Other catecholamines and serotonin were not detected. However, a combination of thin layer chromatography and high performance liquid chromatography suggested the presence of DOPA- and DOPAC-like compounds in nerve-rich tissue. It was not possible to localize dopamine to specific cell types. However, dopamine was not present in swimming motor neurons and B-like neurons. The glyoxylic acid method for catecholamines suggested that a population of small cells had dopaminergic systems. The whole-cell recording technique was employed to examine the physiological action of dopamine on identified neurons in this jellyfish. Dopamine, ranging from 10 nM to 1 mM , applied to cultured swimming motor neurons produced an inhibitory action, namely membrane hyperpolarization and inhibition of firing, by opening potassium channels through D_2-like receptors. The input resistance of cultured neurons was drastically reduced by dopamine, suggesting that dopamine can have an additional inhibitory effect by shunting synaptic currents. In addition, voltage-sensitive potassium channels were opened by dopamine, which may result in changes of spike duration. From these results, it is concluded that dopamine may play a role in *Polyorchis penicillatus* as a neurotransmitter or neuromodulator.

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CHAPTER I
GENERAL INTRODUCTION

Tyger, Tyger, burning bright

In the forests of the night,

What immortal hand & eye

Dare frame thy fearful symmetry?

William Blake

The cnidarians are believed to be the most primitive organisms with a true nervous system (Anderson & Schwab, 1982a). They have attracted considerable attention because of their evolutionary position and the relative simplicity of their neuronal organization. Any information about chemical functioning of the cnidarian nervous system should be useful for reconstructing the evolution of interneuronal communication.

Several cnidarian model systems have been developed with these goals in mind. These include the hydrozoan jellyfish *Polyorchis penicillatus* (Anderson & Mackie, 1977; Spencer, 1978; Spencer & Arkett, 1984), *Aglantha digitale* (Roberts & Mackie,1980) and a scyphozoan jellyfish *Cyanea capillata* (Anderson & Schwab, 1982b). They have been particularly useful for examining synaptic physiology since electrophysiological studies using both pre- and postsynaptic cells are possible *in vivo* and *in vitro*. Physiological data obtained from these preparations and others indicate that many of the basic synaptic properties associated with higher nervous systems are present in cnidarians. The phenomena that have been recorded and which indicate this conventionality include; excitatory postsynaptic potentials (EPSPs), inhibitory postsynaptic potentials (IPSPs), miniature end-plate potentials (mEPPs), facilitation, temporal and spatial summation, and calcium-dependent release of transmitters. Much physiological evidence has accumulated to suggest the existence of chemical transmission in the cnidarian nervous system. Also, ultrastructural evidence for chemical synapses has been found in several cnidarian species (see Westfall's review, 1987). However, the identity of neurotransmitters in the Cnidaria remains enigmatic, despite considerable efforts over the past 30 years by a number of cnidarian neurobiologists. The ultimate goal of the present study is to identify putative neurotransmitters in the nervous system of the Cnidaria.

Polyorchis penicillatus, a hydromedusa, was chosen as the experimental animal for several reasons. Morphological and electrophysiological studies strongly suggested that several identified synapses are conventional chemical synapses in *P. penicillatus*. Ultrastructural studies show that there are dense or clear vesicles on one side of a

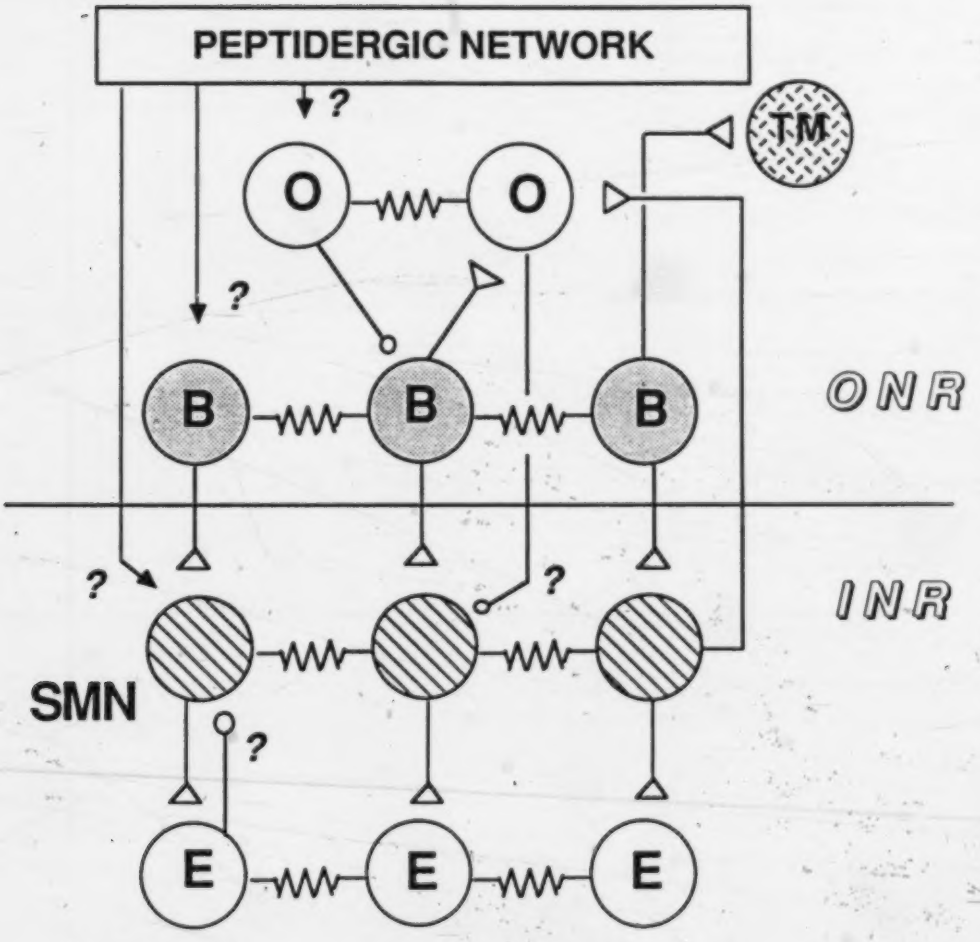
relatively short length of apposed, parallel, electron-dense membrane separated by a synaptic cleft in neuro-neuronal and neuromuscular synapses in *P. penicillatus* (Spencer, 1979). Electrophysiological studies also show that hydrozoan synapses have conventional properties (see Fig. I-1): i) Excitatory postsynaptic potentials follow the presynaptic spikes with a constant delay of about 7 ms between 'B' neurons (bursting neurons carrying photic information) and swimming motor neurons (Spencer & Arkett, 1984), and of 3 ms between swimming motor neurons (SMNs) and overlying epithelial cells (Spencer,1982). ii) This transmission is blocked by high concentrations of Mg^{2+} (Spencer, 1982), suggesting that Ca^{2+}-dependent release may be the mechanism of synaptic transmission in *P. penicillatus* as in the frog neuromuscular junction (del Castillo & Katz, 1954). iii) Injecting current into SMNs does not alter the membrane potential of postsynaptic epithelial cells (Spencer, 1982).

The neuro-neuronal and neuromuscular synapses in *Polyorchis penicillatus* have been more extensively studied than any other cnidarian synapse, since the *in vivo* preparation of *Polyorchis* is amenable to conventional intracellular recording techniques. However, strong electrical coupling between member neurons of a network (Fig. I-1) makes it difficult to apply voltage-clamp techniques to the *in vivo* preparation. Another difficulty that arises when using the *in vivo* preparation relates to pharmacological work. The overlying epithelial cells act as a diffusion barrier which prevents chemicals applied exogenously from reaching target neurons. These problems can be circumvented by the use of a recently developed *in vitro* preparation, a primary culture, in which several types of neurons are isolated (Przysiezniak & Spencer, 1989). Therefore, both physiological and pharmacological approaches which are necessary for identification of transmittters are possible with this *in vitro* preparation of *Polyorchis*.

The case for a particular substance acting as a transmitter can only be built from a number of different lines of evidence. For technical reasons, it is rarely possible to satisfy all the following criteria (Leake & Walker, 1980; Schwartz, 1985) :

Figure I-1. Diagrammatic representation of the organization of identified neuronal networks in the nerve-rings of *Polyorchis penicillatus*.

The swimming motor neurons, SMNs, are located in the inner nerve-ring (INR), while the 'B', 'O', and peptidergic systems are primarily located in the outer nerve-ring (ONR). All these networks consist of electrically-coupled member neurons. Resistor symbols represent electrical coupling between cells within each system. Excitatory chemical synapses are shown by the symbol, Δ, and inhibitory chemical synapses by the symbol, o. Unproven synaptic pathways are maked by '?'. 'E' represents both INR epithelium and epitheliomuscular cells (swimming muscles) overlying SMNs. 'TM' represents tentacle smooth muscle.


PEPTIDERGIC NETWORK
?
TM
O
O
?
B
B
B
ONR
INR
?
?
SMN
?
E
E
E

1. *Presence*

It should be possible to localize the suspected transmitter, either directly by specific staining, fluorescence or autoradiographic markers or indirectly by selectively staining the synthesizing and/or inactivating enzymes involved in its metabolism.

Another commonly employed method is to analyze regional concentrations of suspected synaptic transmitter substances using biochemical techniques. The mere presence of a chemical substance gives no indication of releasibility nor neuroeffectiveness.

2. Release

During stimulation of the presynaptic element, the suspected transmitter should be released from the nerve ending in amounts commensurate with its biologically effective concentration. It should be possible to collect the secreted material for identification and assay (this is often difficult in practice due to the inaccessibility of many synaptic junctions).

3. *Mimicry (biological activity)*

The most important criterion for identification of a suspected transmitter is that the synthetic transmitter should have the same physiological actions as the natural transmitter released on nerve stimulation. Thus, it is essential to establish that exogenous application of the putative transmitter mimics exactly the actions of the endogenous transmitter.

4. *Pharmacological properties*

It is essential that the action of the putative transmitter be altered by pharmacological agents in a predictable way. The effect and time course of agonists should mimic the action of the putative transmitter while antagonists, reuptake blockers and potentiators should influence specifically and predictably the action of the putative transmitter.

5. *Inactivation*

A mechanism should be present for the removal of transmitter material from its site of action, whether this be by a reuptake system or the actions of metabolising enzymes.

Evidence is often collected in a piece-meal fashion until all the criteria are satisfied. However, the evidence which is accumulating on putative transmitter substances in the Cnidaria is especially fragmentary as described in the review by Martin and Spencer (1983): Acetylcholine (ACh), amines, amino acids and peptides have all been proposed as transmitter candidates in the Cnidaria.

Over the past decade, information has accumulated to support the role of peptides as neurotransmitters or neuromodulators in this phylum. Peptides with the carboxy-terminus Arg-Phe-amide are present in neuronal populations within all classes of cnidarians (Grimmelikhuijzen, 1983, 1985; Grimmelikhuijzen & Spencer, 1984) and the biological activities of such peptides have been demonstrated in some cnidarians (McFarlane et al. 1987; Spencer, 1988). On the other hand, the evidence for the existence and functional significance of the conventional transmitters, such as biogenic amines, ACh and amino acids, is still ambiguous and vague.

In spite of the ubiquity of ACh in the animal kingdom and the evidence for its transmitter-like actions in many animals (Table I-1), ACh cannot be regarded as a universal transmitter in the animal kingdom, since evidence of its role as an invertebrate neurotransmitter is not comprehensive. For example, there has been conflicting histochemical and physiological evidence concerning the presence and action of ACh in cnidarians. Bacq (1975) suggested that chemical transmission in the anthozoan *Actinia* is not cholinergic. Kass-Simon & Passano (1978), however, claimed that cholinergic mechanisms are involved in the patterning of ectodermal contraction in *Hydra.* Histochemical studies present an equally confusing picture (Erzen & Brzin 1978, Lentz & Barrnett 1961, and Castano & Rossi 1978). Spencer (unpublished) showed that ACh, its agonists and antagonists produce no behavioral or physiological effects in *Polyorchis.*

Table I-1. Acetylcholine as a neurotransmitter candidate in various invertebrates.

Phylum	Class	Example	Reference
Annelida			
	Hirudinea	leech	Sargent ('77)
Mollusca			
	Gastropoda	snail	Hanley & Cottrell ('74)
	Cephalopoda	octopus	Barlow ('77)
Arthropoda			
	Crustacea	lobster	Hildebrand et al. ('74)
	Insecta	cockroach	Kerkut et al. ('69)
		grasshoppers	Knipper & Breer ('89)
Echinodermata		starfish	Pentreath & Cottrell ('68)

L-Glutamate is the most abundant amino acid in the supraoral sphincter muscle of the sea anemone *Actinia equina* (Carlyle, 1974). On the other hand the level of aspartic acid was low and γ-aminobutyric acid (GABA) was not detected. The presence of glutamate in muscle tissues does not necessarily indicate a neurotransmitter role, since L-glutamate is an amino acid which has a role in metabolism. In fact, glutamate appears to be present in all groups of animals. Anderson & Spencer (1989) reported that most common excitatory amino acids do not generate any responses from one of the two neurons forming a chemical synapse in *Cyanea capillata*. Recently (Anderson & Trapido-Rosenthal, 1990) presented evidence showing that taurine could be the transmitter at this synapse. No attempt has been made to investigate GABA in cnidarians, although this is an important transmitter in several major phyla (Table I-2), such as arthropods and chordates.

Of the putative neurotransmitters, besides peptides, that have been tested in various cnidarian preparations, the best evidence suggests that biogenic amines may be transmitters. Most of our knowledge of the biogenic amine systems in cnidarians comes from biochemical and histological studies, however, the precise functions served by the catecholamines are, as yet, poorly known. Several studies have used extracellular recording techniques to examine the action of biogenic amines, but none have allowed an understanding of these actions at the cellular level (Ross, 1960a,b; Lentz & Barrnett, 1963; Schwab, 1977; Anctil et al. 1982; Anctil, 1989).

Despite some danger of following a false trail, monoamines were chosen as the first substances for investigation for the following reasons;

1. Monoamines are widely distributed throughout the animal kingdom and in many cases these have been shown to have transmitter-like roles (Table I-3). Therefore, it is possible that they are a primitive group of transmitter substances and appeared early in the evolution of nervous systems.
2. There are preliminary data supporting the presence and activity of monoamines in the Cnidaria.

Table I-2. Amino acids as neurotransmitter candidates in various invertebrates.

Phylum	Class	Example	Amino acids	Reference
Cnidaria				
	Anthozoa	sea anemone	Glu.	Carlyle ('74)
	Scyphozoa	jellyfish	taurine	Anderson & Trapido-Rosenthal('90)
Annelida				
	Hirudinea	leech	GABA, Gly	Sargent ('77)
			Glu.	James & Walker ('78)
	Oligocheta	earthworm	GABA	Ito et al. ('69)
Mollusca				
	Gastropoda	snail	Glu	Cottrell et al. ('72)
			GABA	Yarowsky & Carpenter ('77)
	Cephalopoda	squid	Glu.	Miledi ('67)
		squid	Glu.	De Santis & Messenger ('89)
Arthropoda				
	Crustacea	crab	Glu	Florey & Woodcock ('68)
		lobster	Glu	Colton & Freeman ('75)
		crayfish	Glu	Onodera & Takeuchi ('75)
		crab	GABA	Florey & Rathmayer ('72)
		lobster	GABA	Otsuka et al. ('66)
		crayfish	GABA	Takeuchi & Takeuchi ('65)
		lobster	Asp.	Shank et al. ('75)
	Insecta	locust	Glu.	Dowson & Usherwood ('73)
		cockroach	GABA	Kerkut et al. ('69)
		grasshopper	Glu.	Usherwood & Machili ('68)
	Chelicerata	horse-shoe crab	GABA, Glu	Walker & James ('78)
Echinodermata		sea urchin	GABA	Florey et al. ('75)

** GABA, γ-aminobutyric acid; Glu, glutamate; Asp, aspartate; Gly, glycine.*

Table I-3. Monoamines as neurotransmitter candidates in various invertebrates.

Phylum / Class	Example	Amines DA	NE	5HT	OCT	Reference
Annelida						
Hirudinea	leech	+	?		+	Stuart et al. ('74)
	leech			+ +		Kerkut & Walker ('67)
	leech			+ +	+ +	Hashemzadeh-Gargari & Friesen ('89)
Oligocheta	earthworm	+	+	+		Ehinger & Myhrberg ('71)
	earthworm				+	Robertson ('75)
	earthworm			+ +		Gardner & Cashin ('75)
Mollusca						
Cephalopoda	octopus	+	+	+	+	Juorio ('71) Juorio & Molinoff ('74)
	cuttlefish	+	+	+	+	Kling & Schipp ('87)
Gastropoda	snail	+ +	+			Berry & Pentreath ('78)
	snail			+ +		Loker et al. ('75)
	snail				+ +	Batta et al. ('77)
	snail	+ +		+ + +		Buckett et al. ('90)
Arthropoda						
Crustacea	crab	+ +	-		+ +	Kerkut et al. ('66)
	lobster	+ +	-			Kushner & Maynard ('77)
	lobster			+ +		Cooke ('66)
	lobster				+ +	Evans ('78)
Insecta	cockcroach					
	s. gland	+ +	-			Fry et al. ('74)
	brain	+ +	+ ?			Frontal ('78)
	locust					
	s. gland	+ +	-			Klemn ('72)
	s. gland				+	Pannabecker & Orchard ('86)
	nerve	+ +	-			Klemn ('72)
	nerve			+		Peters et al. ('87)
	brain	+ +	+ ?	+ +	+	Hiripi & S.-Rozsa ('73)
	moth	+ +	-			Robertson ('75)
	tick	+ +	+ ?			Kaufman ('77)
Echinodermata						
	starfish	+	+			Cottrell ('67)
	starfish	+	+		+	Juorio & Robertson ('77)

* CRITERIA. Presence, Synthesis, Mimicry (physiological activity), Pharmacological behavior. Numbers of "+" symbol represent those of the above criteria to be satisfied: e.g. +, presence; ++, presence & mimicry, etc.; +?, present but physiologically inactive.; -, no presence; ?, no convincing data, etc.
DA, dopamine; NE, norepinephrine; 5HT, serotonin; OCT, octopamine.

3. Monoamines have been studied extensively in other groups and a wide range of techniques is readily available.

It was apparent from previous studies that to obtain useful data it would be preferable to take a multidisciplinary approach applying as many techniques as feasible. The major amines present in *Polyorchis* were first examined using several analytical techniques; TLC, HPLC, and GCMS. The physiological effects of the amine candidates on cultured SMNs of *Polyorchis penicillatus* were then investigated electrophysiologically. Details of the chemical analyses and the electrophysiological experiments are described in Chapters II and III, respectively. In Chapter IV, the precise nature of the interaction of amines with receptors is examined using a pharmacological approach. Chapter V consists of my concluding remarks.

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CHAPTER II.

DOPAMINE IS PRESENT IN NERVE-RICH TISSUES OF THE HYDROZOAN JELLYFISH *Polyorchis penicillatus*.

Even though the results could be more exciting, I hope that some journal will accept it as a cautious and careful investigation within a mine-field where artefacts are as plenteous as jellyfish in the Skagerakk during August.

A letter from Dr. M. Carlberg

INTRODUCTION

One of the important criteria for establishing a substance as a neurotansmitter is that a substance must be present in and released from a neuron at a sufficiently high concentration to produce a predicted effect. The main purpose of this chapter is to examine the presence of biogenic amines in the hydromedusa, *Polyorchis penicillatus*, since biogenic amines are one of the strongest candidate substances for neurotransmission in the cnidarian nervous system (refer to the General Introduction).

Since Östlund (1954) discovered an unknown catechol derivative in the sea anemone *Metridium dianthus* using paper chromatography, there have been several analytical studies identifying biogenic amines in the cnidarian nervous system (Table II-1). However, many of these biochemical studies have been notoriously difficult to interpret. For example, Lenique et al. (1977) demonstrated dopamine (DA), 3,4-dihydroxyphenylalanine (DOPA) and serotonin using thin layer chromatography (TLC) in *Metridium senile* but these results can be criticized since the colors of fluorescence of reference reagents were different from those of sample extracts with similar R_f values. Carlyle (1969) reported that compounds resembling dopamine and DOPA were detected in only eight of nineteen experiments in *Actinia equina* using paper or thin layer chromatography and that catechol-4, which was named by Östlund (1954), was not detected with this assay system. From these earlier results it is difficult to conclude that catecholamines are present in cnidarian tissues because the analytical methods used were rather inefficient with regard to both separation and detection. Employing high performance liquid chromatography with electrochemical detection (HPLC-ED), which is more efficient, sensitive and specific than TLC, Carlberg (1983) could find neither catecholamines nor serotonin in both *Metridium senile* and *Tealia felina* (Anthozoa). However, he did detect DOPA and its derivatives. These results directly contradict those of Lenique (1977). On the other hand, Venturini et al. (1984) reported the presence of norepinephrine, dopamine, serotonin and unidentified compounds in the hydrozoan *Chlorohydra viridissima* using HPLC. Also, in a later study, Carlberg and Rosengren (1985) showed the presence of catecholamines in the scyphozoan, *Cyanea*, and the athecate hydrozoans *Hydra* and *Tubularia*. In summary,

Table II-1. Chemical Analysis of Biogenic Amines in Cnidarians.

Östlund E	*Metridium dianthus*	PC*	Catechol-4 (Low R_f)
(1954)			No CAs.
Mathias AP et al.	*Calliactis parasitica*	PC	5HT.
(1960)	*Metridium senile*	PC	No 5HT.
	Anemonea sulcata	PC	No 5HT.
Welsh JH	*Hydra oligactis*	PC/F	5HT.
(1960)	*Sagartia luciae*		5HT.
	Metridium senile		5HT.
Carlyle RF	*Actinia equina*	TLC*	DOPA>DA>NE>EN(?)
(1969)			Unknown (High R_f)
			No Catechol-4.
			No 5HT, ACh.
Elofsson R et al.	*Metridium senile*	F/GCMS	DOPA
(1977)	*Tealia felina*		No DA, NE, 5HT.
Lenicque PM et al.	*Metridium senile*	TLC#/F	DA, DOPA, tryptamine,
(1977)			Unknown (Low R_f)
Carlberg M	*Metridium senile*	HPLC-ED	DOPA, 5-OH-DOPA
(1983)	*Tealia felina*		5-SH-DOPA, No CAs.
Venturini G et al.	*Chlorohydra*	HPLC-ED	NE, DA <5HT
(1984)	*viridissimia*		CA-like, 5HT-like.
Carlberg & Rosengren	17 cnidarian species	HPLC-ED	NE, DA.
(1985)			
De Waele J-P et al.	*Renilla köllikeri*	HPLC-ED	NE>DA>EN, DOPA?
(1987)		REA	DA>NE>EN.
Chung JM et al.	*Polyorchis*	HPLC-ED*	DA, NE-like, DOPA?
(1989)	*penicillatus*	GCMS*	DA, No NE & EN.

PC, paper chromatography; TLC, thin layer chromatography; F, fluorometry; HPLC-ED, high performance chromatography with electrochemical detection; GCMS, gas chromatography-mass spectrometry; REA, radioenzymatic assay.

* Alumina extracts were used.

Organic solvent extracts were used.

CA, catecholamine; EN, epinephrine; 5HT, serotonin; ACh, acetylcholine; DA, dopamine; NE, norepinephrine; DOPA, 3,4–dihydroxyphenylalanine.

some investigations have demonstrated the presence of biogenic amines (Welsh, 1960; Carlyle, 1969; Lenique et al., 1977; Venturini et al., 1984; Carlberg and Rosengren, 1985) in several cnidarian species while other studies have denied the presence of known catecholamines (Östlund, 1954; Elofsson et al., 1977; Carlberg, 1983). This discrepancy may be due to the seasonal- or the species-variation in amine content, the various sensitivities of the different methods used, and perhaps the presence of a substance in cnidarians which inhibits the normal migration of catecholamines on chromatographs (Carlyle, 1969).

I have found that samples extracted from *Polyorchis penicillatus* with acid-activated alumina gave complex chromatograms with several unknown peaks often having retention times that correspond to, or overlap with, the peaks of interest such as dopamine, norepinephrine, epinephrine, their acidic metabolites, and DOPA. It must be remembered that these results were obtained using whole animals. This is one of the weakest aspects of any analytical-chemical approach using cnidarian tissues, since it is normally difficult to isolate nervous elements. In this study, three distinct types of tissues with different concentrations of nervous tissue were used under the assumption that a substance working as a neurotransmitter would be present in higher concentrations in "nerve-rich" tissues than other tissues.

To demonstrate clearly the presence of biogenic amines in *Polyorchis penicillatus*, it is preferable to use more than one analytical method. In the present study, I have quantitatively demonstrated the presence of dopamine in *Polyorchis penicillatus* using HPLC-ED and have confirmed its presence qualitatively using gas chromatography combined with mass spectrometry (GCMS). In addition, I suggest that several unknown compounds, probably related to catecholamines, are present in *Polyorchis penicillatus*.

MATERIALS AND METHODS

Methodological Considerations.

As described in the Introduction, both qualitative and quantitative data on the presence of biogenic amines in cnidarians are quite variable from laboratory to laboratory and from species to species. These variations may be explained by: (1) The high water content of cnidarian tissue. The water content of human brain is 74.8% (Harth, 1983) while the water content of *P. penicillatus*, is 83 to 97% (Fig. II-1). Therefore, there is a tendency to underestimate concentrations in cnidarians. (2) The level of catecholamines reported in the Cnidaria is lower than that in the mammals. For example, the levels of catecholamines, reported for cats and dogs, in ganglia is 1-2 µg/g dopamine and 5-40 µg/g norepinephrine (Clark, 1985); while catecholamine levels are in the ng/g range in most of the Cnidaria (Carlberg & Rosengren, 1985). This may be related to the observation (Spencer, 1979; Satterlie & Spencer, 1983; Satterlie, 1985; Anderson & Spencer, 1989) that the number of vesicles in cnidarian neurons are far fewer than that in other animals. Thus, the extremely low levels of endogenous compounds potentially involved in neurotransmission require that efficient separation techniques and sensitive detection devices are used.

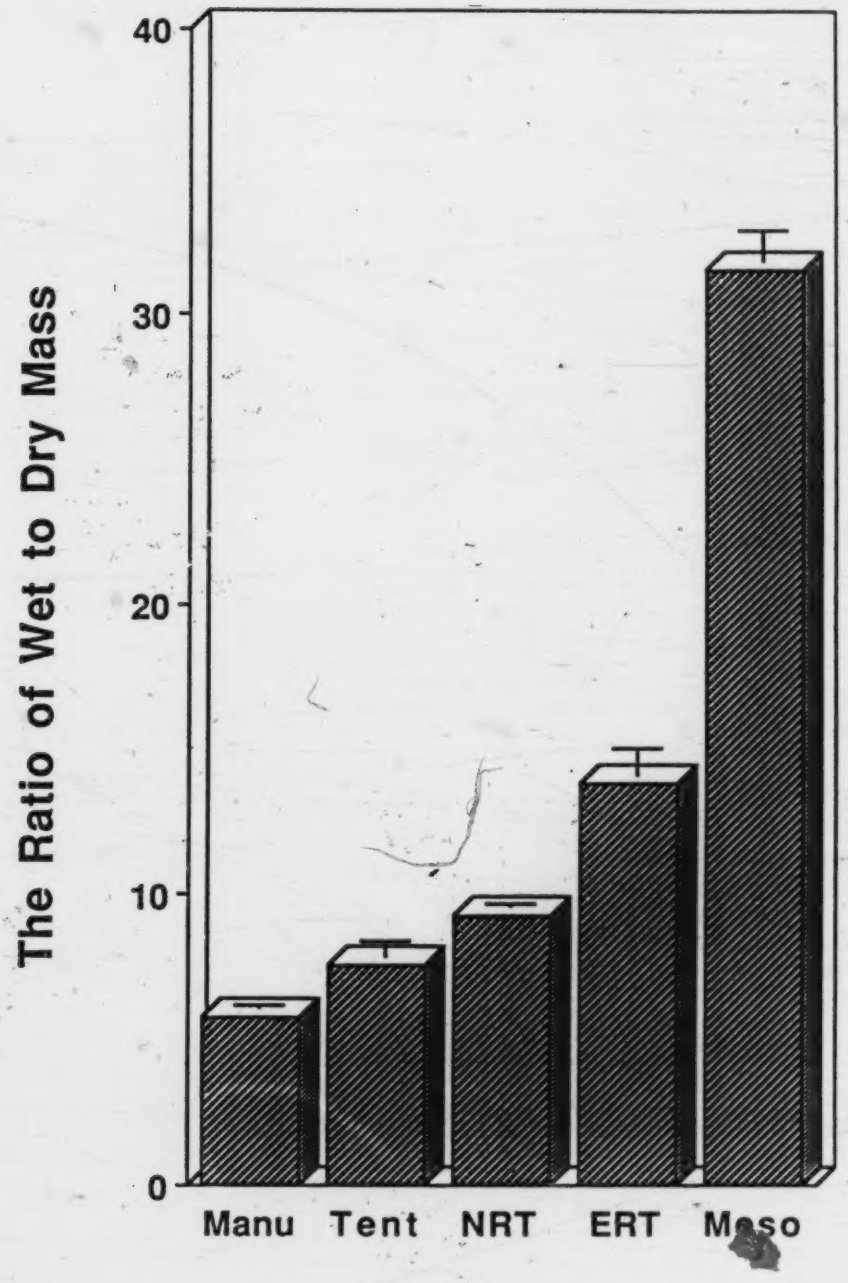
The considerable attention focused on the role of catecholamines in a number of neurological disorders has prompted the development of a number of analytical methods. These techniques include radioenzymatic assays (REAs; Ben-Jonathan & Porter, 1976), double-isotope derivative analyses (Engleman et al., 1968), gas chromatography alone (Wilk et al., 1965) or coupled with mass spectrometry (Gelpi et al., 1974), and HPLC with fluorometric (Krstulovic & Powell, 1979) or electrochemical detection (ED; Refshauge et al., 1974).

Radioenzymatic procedures, which employ the specific radiolabelling of the catechol moiety with S-adenosylmethionine and catechol-O-methyltransferase (COMT), possess the requisite sensitivity and specificity. However, they require multiple sample handling steps, enzyme preparations, and labelled compounds which makes them tedious, time-consuming and expensive for routine analysis. In addition, the enzymatic reaction may be inhibited by the sample components. An unidentified compound which is

Figure II-1. Relative water content of the tissues in jellyfish.

Each tissue sample was pooled from 20-30 animals, and the surface water was removed with Whatman filter papers and the tissue sample weighed (wet mass). The tissue samples were immediately frozen at –25 °C (dry ice with acetone) and then desiccated in a freeze-dryer (Freezemobile 6, VirTis Comp. Inc.) for three days. The dried sample was weighed (dry mass) and stored in a desiccator at –20 °C until assayed. The relative water content of each tissue was determined by comparing its wet mass with dry mass (N=5). *Error bars, SEM.*

Manu, manubrium; Tent, tentacles; NRT, nerve-rich tissue; ERT, endoderm-rich tissue; Meso, mesoglea.


The Ratio of Wet to Dry Mass
40
30
20
10
0
Manu
Tent
NRT
ERT
Meso
Various Tissues

structurally related to catecholamines can also be a potential source of error. The double-isotope derivative methods also suffer from similar problems.

Earlier gas chromatographic methods employing electron-capture detection have not achieved wide popularity due to the problems associated with derivatization of complex matrices and the vagaries of the electron-capture detector. In this procedure catecholamines and their metabolites are treated with halogenated anhydrides and the resulting volatile derivatives possess excellent electron-capturing properties (Clarke et al. 1966). Although the absolute sensitivity of this detector is high, the usable sensitivity is usually decreased due to the sample background arising from the derivatization reagents and contaminants from the sample matrix and solvents. More recently, the tandem operation of gas chromatography and mass spectrometry has emerged as an extremely sensitive method, having at least three distinct advantages; (a) the sample does not have to be highly purified when only certain molecular ions are of interest, (b) deuterated reference compounds, which behave identically as their non-deuterated analogues, can be used and thus high precision can be achieved, (c) with halogenated anhydrides as derivatizing reagents, excellent fragmentation patterns with prominent molecular ions are obtained. However, this method still requires derivatization. Another great disadvantage is that this method requires expensive instrumentation and a high level of technical expertise.

HPLC, particularly in its reversed phase mode, where the mobile phase is relatively polar and the stationary phase relatively non-polar, offers another approach for circumventing the problems commonly associated with other methods of analysis. This technique is ideally suited for the determination of thermally labile biological compounds since it affords high resolution and rapid analysis without prior derivatization. Therefore, HPLC was chosen as a primary tool in this study.

A. HPLC

1. Modes of HPLC.

Today, analytical HPLC is nearly always done with microparticulate column packings, since they have high efficiency and large sample capacity. Efficiency of the

column is increased by minimising the time for mass transfer due to the fine particle size and the sample capacity is increased because of the large surface area. Microparticulates are small porous particles, usually spherical or irregular silica, with nominal diameters of 3, 5, or 10 μm. In bulk, the appearance of a microparticulate silica resembles that of fine talcum powder.

Microparticulate silicas have been used in a number of different ways in HPLC: (a) as adsorbents (b) as supports for stationary liquids in partition chromatography (c) as bonded phases (d) as materials for exclusion chromatography. Bonded phase chromatography is experimentally much easier than adsorption or liquid-liquid partition. It is more versatile, faster, and has better reproducibility than the other modes. In a bonded phase, the highly polar surfaces of the silica are altered by the chemical attachment of different functional groups. These attached groups can be nonpolar (eg, C-18), polar ($-NH_2$, -CN) or ionisable (sulphonic acid, quaternary ammonium). The introduction of ionisable groups produces bonded phases with ion-exchange properties. Figure II-2 shows how an HPLC method was chosen in my experiment on the basis of the solubility of the sample and the sort of functional groups that it contains. The figure shows only silica or modified silica stationary phases. It can be seen from the figure that almost any separation can be achieved by reverse phase chromatography using a bonded silica stationary phase. This is the mode that I used; it is faster, cheaper and experimentally easier than the alternatives.

The reverse-phase mode, although having several advantages, is not without limitations. Among the more important of these are: (a) The mechanism of separation is still poorly understood; (b) for silica bonded phases, stable columns can be maintained only over a pH range between about 3 and 8. Below pH 3, the bonded group may be removed, and above pH 8 the silica is appreciably soluble in the mobile phase. However, such a limited pH range is not a problem for detection of catecholamines, since catecholamines are notoriously unstable at elevated pH; and (c) the presence of unreacted silanol groups on the silica surface can often cause poor peak shape and nonreproducible behaviour between columns, due to solute adsorption.

Amines are weak bases and at the pH used (3 to 5) will be completely protonated. Because the protonated bases are very polar compounds, they are adsorbed strongly by unreacted silanol groups, causing excessive retention and severe peak tailing. There are

several ways to approach the problem. One is to add a high concentration (relative to the concentration of the solutes) of a competing base to the mobile phase. Because of its relatively high concentration, the competing base is preferentially adsorbed by the silanol groups, thus minimising the adsorption of the other bases. Other possible ways of achieving separation would be to use ion-suppression or ion-pairing techniques. Ion-suppression is used for the chromatography of weak acids or bases. The principle is that the ionization of an acid or the protonation of a base is suppressed by adjusting the pH, and then the sample is run on a reverse-phase column using methanol or acetonitrile plus a buffer solution as the mobile phase. The technique is preferable to ion-exchange because the C-18 column has higher efficiency and equilibrates faster than ion-exchange columns. Ion-pairing techniques are generally used to separate weak acids and bases but they also find application in the separation of other ionic compounds. An ionised compound (A^+_{aq}) that is water soluble can be extracted into an organic solvent by using a suitable counter ion (B^-_{aq}) to form an ion-pair, according to the equation:

$$A^+_{aq} + B^-_{aq} \longleftrightarrow (A^+B^-)_{org}$$

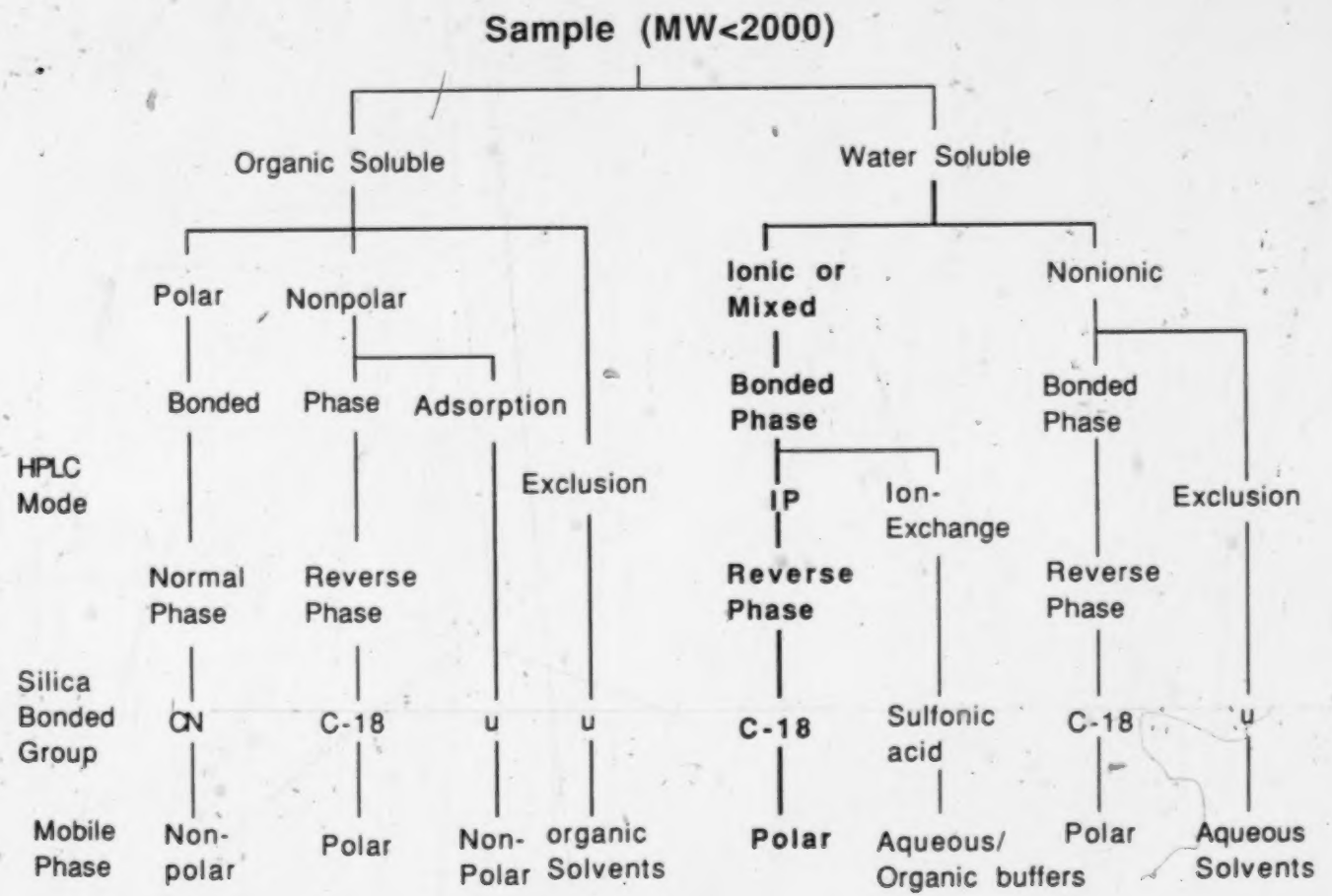
The ion-pair (A^+B^-) behaves as if it is a nonionic polar molecule, soluble in organic solvents. By choosing a suitable pairing ion and adjusting its concentration, the ion A^+ can be efficiently extracted into an organic phase. Similarly, anions can be extracted by using a suitable cationic pairing ion. I used reverse-phase separation on a C-18 column, with an ion-pairing reagent such as octyl sulfate added to the mobile phase. The methods I chose are in bold in Fig. II-2.

2. Detection.

To achieve efficient and rapid separation of catecholamines and their metabolites, a detection system must be used that is both sensitive and compatible with the chromatographic system. Only two detectors, the flurometric and the electrochemical, fulfilled these requiments. Both detectors are quite sensitive to catecholamines; however, an electrochemical detector (ED) was employed for this study. The types and the operation of electrochemical detectors are illustrated in Appendix 1.

Figure II-2. Choice of HPLC mode.

MW, molecular weight; IP, ionic pairing; u, unmodified.


Sample (MW<2000)
Organic Soluble
Water Soluble
Polar
Nonpolar
Ionic or Mixed
Nonionic
Bonded
Phase
Adsorption
Exclusion
Bonded Phase
Bonded Phase
HPLC Mode
IP
Ion-Exchange
Exclusion
Normal Phase
Reverse Phase
Reverse Phase
Reverse Phase
Silica Bonded Group
CN
C-18
u
u
C-18
Sulfonic acid
C-18
u
Mobile Phase
Non-polar
Polar
Non-Polar
organic Solvents
Polar
Aqueous/ Organic buffers
Polar
Aqueous Solvents

3. Materials for HPLC analysis.

Dopamine (DA), epinephrine (EN), norepinephrine (NE), epinine, 5 hydroxytryptamine (5HT; serotonin), tryptamine, octopamine hydrochloride, 3,4-dihydroxybenzylamine (DHBA), 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxyphenylglycol (DHPG), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (HIA), tyrosine (Tyr), and tris(hydroxymethyl)aminomethane (TRIS) were obtained from Sigma (St. Louis, MO, USA): sodium octyl sulfate (SOS) was from Kodak (Rochester, NY, USA): Other chemicals were HPLC or reagent grade and were obtained from local suppliers.

4. Methods.

1) HPLC-ED system.

Apparatus I. A model 6000 liquid chromatograph was equipped with a Rheodyne 7125 injection valve with a 200 µl sample loop, an Econosphere RP-18 column (5 µm particle size, 250x4.7 mm I.D.), and a BAS LC-4B amperometric detector with a TL-5 glassy carbon electrode maintained at a potential of 0.75 V vs an Ag/AgCl reference electrode with a sensitivity of 2 nA/V.

Apparatus II. A Waters M-45 solvent delivering system was equipped with a U6K injector with a biophase ODS RP-18 column (5 µm spheres, 250x4.6 mm I.D.) from Bioanalytical Systems Inc., and a BAS LC-4A detector with an applied potential of 0.8 V. For injection into both liquid chromatographs, Hamilton 801 or 825 syringes were used.

Mobile phase for system-I. The components of the mobile phase were as follows: 55 mM monobasic sodium phosphate, 0.85 mM SOS, 7 mM disodium EDTA, and 9% acetonitrile.

Mobile phase for system-II. The components of this mobile phase consisted of 50 mM monobasic sodium phosphate, 30 mM citrate buffer, 0.1 mM disodium EDTA, and 25% methanol.

pH adjustments were made using conc. phosphoric acid. Both mobile phases were finally vacuum-filtered through a 0.22 µm membrane filter (Millipore). The flow rate was 0.5 ml per minute.

2) Preparation of standard solutions.

Working standard amine solutions of 80 nM were prepared by dilution of stock solutions immediately prior to use. The stock solutions of reference amines and internal standards (1 mM) were prepared monthly in 0.02 N HCl containing 0.54 mM EDTA sodium salt. These solutions were stored in dark bottles at about 4 °C.

3) Sample preparation.

The determination of catecholamines in tissue samples is often plagued by the problems associated with post-mortem changes. The extent of these changes depends upon the time that elapses between death and sample preparation (Carlsson & Winblad, 1976), as well as the temperature at which samples were stored prior to processing (Wilk & Stanley, 1978). The optimal conditions for assesment of catecholamine levels in a sample requires storage of the tissue sample at 4 °C and immediate homogenization after dissection.

Scuba divers collected *Polyorchis penicillatus* from Bamfield Inlet on the west coast of Vancouver Island, Canada. The jellyfish (Fig. II-3A) were then airfreighted to Edmonton. These animals were kept in cooled, recirculated artificial sea water (10-12 °C). They were sometimes fed with brine shrimp. All animals were starved for a minimum of 2 days before use. The following tissue samples were dissected from live animals (refer to Fig. II-3B):

Nerve-rich tissue; consisted of a strip of tissue which included one third of the width of the velum and some ring-canal tissue in addition to all of the inner and outer nerve-rings. Hence, it contained nervous tissue, swimming muscle, gut and mesoglea (see Spencer, 1979).

Endoderm-rich tissue; contained strips of the radial canal with the radial muscles and their nerves stripped off. Hence, it contained gut, mesoglea, swimming muscle and perhaps a small amount of nervous tissue (see Spencer, 1979).

Mesoglea; consisted of pieces of umbrella mesoglea without any attached cellular tissue.

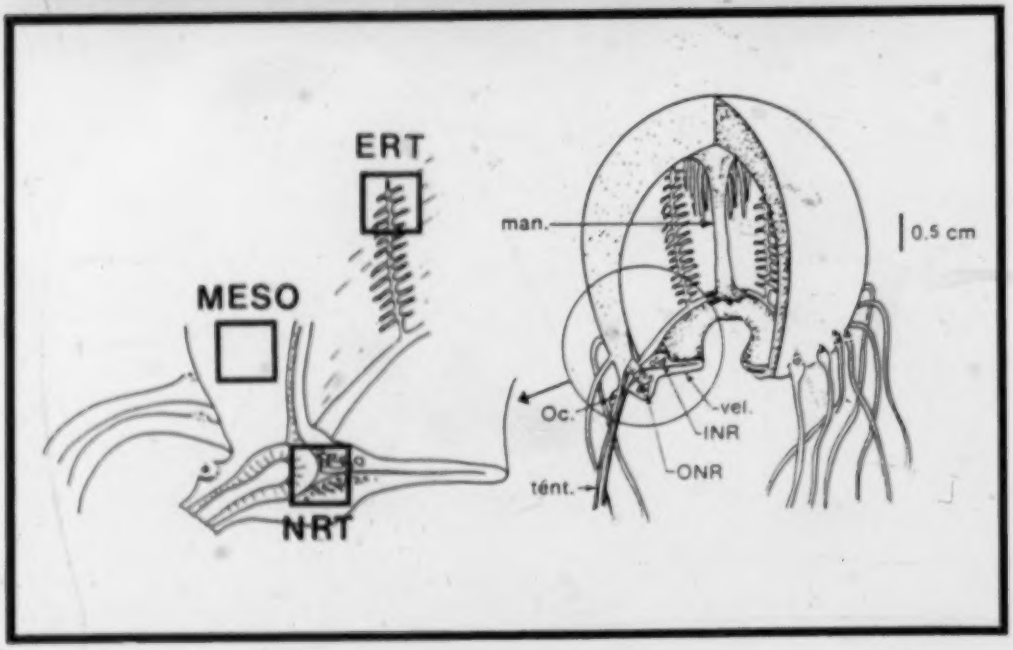
Figure II-3. Diagrammatic representation of *P. penicillatus*.

A. Photograph of *Polyorchis penicillatus* in situ in Bamfield Inlet. (Photographed by Mr. P. Marko.)

B. Schematic illustration showing the major anatomical features of *Polyorchis penicillatus*. The cut-away shows the locations of the dissected tissue used for biochemical analysis. (From Arkett & Spencer, 1986)

A

B



Samples were pooled from 5 to 8 animals as required to give sufficient tissue (100-200 mg wet mass), then the surface water was removed and the sample weighed. After weighing, the tissue samples were immediately frozen at –25 °C. One ml of freshly prepared, ice-cold 0.1 N perchloric acid containing 1% sodium metabisulphite, and internal standard (DHBA; dihydroxybenzylamine) were added to 100-200 mg of the frozen tissue. The samples were homogenized, placed in an ice bath for 10 min, and then centrifuged at 3600 x *g* for 1 h at 4 °C. The catecholamines were extracted with acid-activated alumina, baked at 120 °C for 2 h, following the procedure described by Cyril (1985). The supernatant was transferred to another tube containing 50 mg of activated alumina. After adding 1.5 M TRIS buffer containing 54 mM EDTA (pH 8.6), the tube was vortexed for 5 min and then centrifuged for 30s at 1000 x *g*. The solution was removed by vacuum aspiration and the alumina was washed twice with double-distilled, deionized water (pH to 7.0). Catecholamines were eluted with 100 µl of 0.1 M or 0.5 M HCl.

4) Calculations of catecholamine concentrations.

Figure II-4 demonstrates standard curves for both norepinephrine and dopamine. They show that the current responses of the detector were linearly related to the amount of catecholamines present, within a given range. Although the liquid chromatograph system worked with high reproducibility, both qualitatively and quantitatively, catecholamines were quantified using the "internal standard" method (Gerlo & Malfait, 1985) since the level of catecholamines in biological samples may not be accurately determined without considering either the loss of amines during alumina extraction or the matrix effect. The amounts of the internal standard (DHBA) and standard samples used were within the range where the response of the detector was linear.

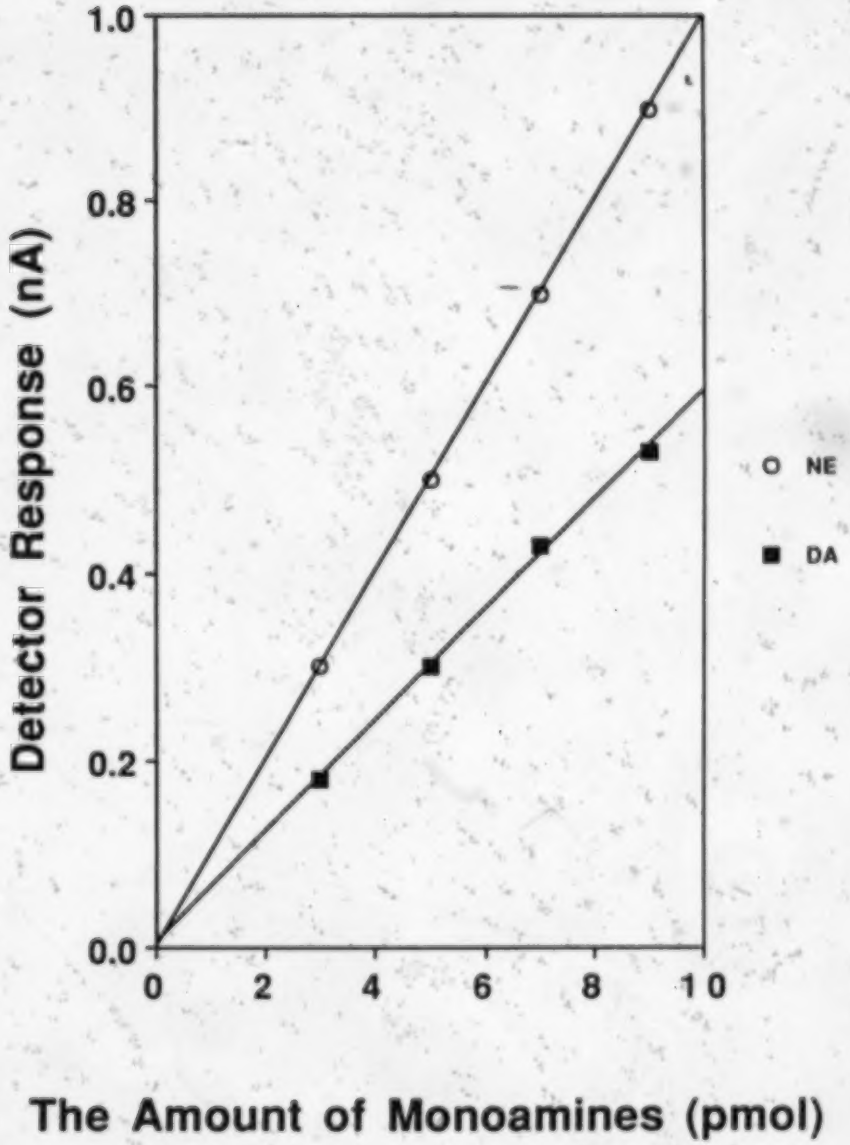
Sample concentrations were calculated by comparing peak height ratios (relative to DHBA) of samples with peak height ratios of the unextracted standards. For example, Concentration of DA in a sample =

$$\frac{\text{peak height ratio DA/DHBA for sample}}{\text{peak height ratio DA/DHBA for standard}} \times [\text{I.S.}] \times K$$

where [I.S.] is the concentration of the internal standard DHBA added to the sample and K is the calibration factor due to the matrix effect.

Figure II-4. Standard curves of catecholamines.

A series of solutions of the standard compounds such as dopamine (DA,■) and norepinephrine (NE,O) were chromatographed using LC system II (see materials and method) and a plot of peak current amplitude versus concentration was constructed. The plots were linear (r^2 = 1.000 and 0.997 for NE and DA, respectively, within a given concentration range), indicating that the LC system operated effectively and the injection volumes were reproducible.


1.0
0.8
0.6
0.4
0.2
0.0
Detector Response (nA)
0
2
4
6
8
10
NE
DA
The Amount of Monoamines (pmol)

B. TLC

1. Materials for TLC analysis.

One ml of freshly prepared, ice-cold 0.1 N perchloric acid containing 1% sodium metabisulphite was added to 0.05 g of the dried tissue. The samples were homogenized, placed in an ice bath for 10 min, and then centrifuged at 3000 x *g* for 30 min at 4 °C. The supernatants were used in TLC analysis. Chemicals used were identical to those described previously.

2. Methods.

Several investigators (see Table II-1) have demonstrated the occurrence of either monoamines or unidentified catechol derivatives in cnidarian tissues using either paper chromatography or TLC. A TLC method was employed in order to compare these results with previous studies and with the HPLC results.

TLC was performed on commercially pre-coated 20 x 20 cm, 0.25 mm silica gel TLC plates (Whatman Co.), using n-butanol-acetic acid-water (volume ratio, 60 : 20 : 20) as the solvent. The elution procedure lasted 4 h at room temperature. The front was allowed to advance 16 cm from the base line. Two development procedures were used: i) The bands were developed by spraying the plates with a solution of 2% paraformaldehyde in absolute ethanol and then baking them in an oven at 150 °C for 20 min. Fluorescent bands were generated by UV light. ii) Control plates, which were not treated with paraformaldehyde solution, were completely dried under an air-stream. Samples of silica gels at specific regions were obtained by scratching them off with a razor. They were then placed in 0.5N HCl or methanol solution. After removal of the silica gel from these solutions with centrifugation at 3000 x *g* for 30 min, the supernatants were introduced to the HPLC.

C. GCMS

To use retention data alone for the identification of unknown solutes would be rather like trying to identify an unknown organic compound simply by measuring its melting

point or boiling point. Many different solutes will have essentially identical retention times for a particular set of conditions. To overcome this problem, two HPLC systems with different sets of conditions were employed in my study to identify catecholamines in *P. penicillatus*. For additional confirmation of the identities of the HPLC peaks, the more powerful method of mass spectrometry was used.

1. Advantages and limitations of GCMS.

In principle, the direct combination of liquid chromatography and mass spectrometry (LCMS) would be one of the best for the identification of the compounds in a biological sample. However, the sensitivity of the LCMS for amine compounds is still much lower than that of gas chromatography combined with mass spectrometry (GCMS). The GCMS technique offers the greatest sensitivity presently available for the detection of trace organic compounds such as catecholamines. The capillary columns provide highly inert systems to assay labile substances. The capillary columns are so efficient that substances closely related in complex samples can be separated. The technique of selected ion monitoring (SIM), where the mass spectrometer focuses on only a few pre-selected mass-characteristics of the compound rather than scanning the entire mass range, is now used extensively because it permits a considerable relaxation in sample purification requirements. It also provides confirmation of the identity of a compound when two or more compounds co-elute (Jacob et al. 1984). The sensitivity and specificity of GCMS can be increased further by the use of new ionization methods, such as negative ion chemical ionization (NICI). The chemical ionization technique avoids the extensive fragmentation of the molecular ions and thus improves the detectability of molecular ions relative to that obtained with electron impact ionization. The potential sensitivity of NICI GCMS has been demonstrated by Hunt and Crow (1978) who achieved a 10- to 100-fold sensitivity enhancement for electron capturing compounds over electron impact (EI) GCMS. Therefore, NICI GCMS was employed in this study. Further explanation for both the mode of operation of the GCMS and the principle of NICI are given in Appendix 2.

2. Materials for GCMS analysis.

Trifluoroacetic anhydride (TFAA) was from General Intermediates of Canada and

other chemicals used were identical to those described previously.

3. Methods.

1) GCMS system.

The GCMS system consisted of a gas chromatograph (Varian, Vista 6000) equipped with a SPB-5 fused silica capillary column (30 m length, 0.25 μm film thickness) and a VG 7070E mass spectrometer equipped with a dual electron impact (EI)/chemical ionization (CI) source with the capability of analyzing positively and negatively charged ions. The end of the capillary column was inserted directly into the ion source of the mass spectrometer. Helium was used as the carrier gas with a flow rate of about 1.5 ml per min. The temperature of the GC oven was kept at 100 °C initially and increased to 290 °C by 10 °C per min. The injector and the interfacial region between the gas chromatograph and mass spectrometer were maintained at 240 °C.

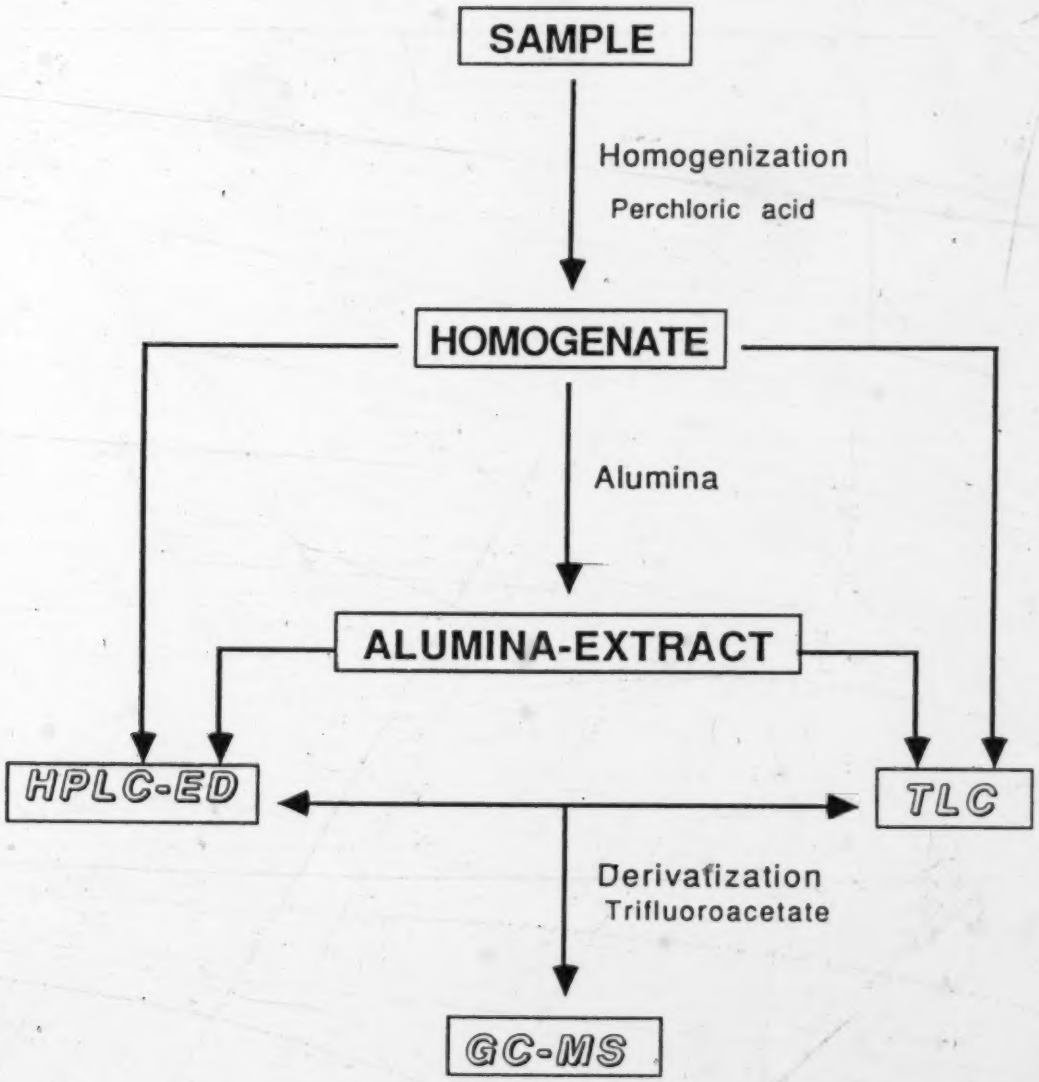
The mass spectrometer was operated at a resolution of R=1000. The ionization energy was 150 eV and emission current was 5 mA for the CI mode. The ion source temperature was maintained at 190 °C. The mass spectrometer was set to scan mass unit 130 to 700 since there was considerable interference by the iodide ion at mass unit 127 in the NICI mode. To take NICI spectra, methane was used for the reagent gas. A PDP-11/73 computer was used to acquire the data.

2) Sample derivatization.

Alumina extracts or given standard solutions were evaporated with a gentle stream of nitrogen under reduced pressure. One hundred μl of ethyl acetate and 100 μl of trifluoroacetic acid (TFAA) were added to the dried residue, and the mixture was then allowed to stand to react for 1h at room temperature. The mixture was evaporated again to remove excess reagents with a stream of nitrogen and 10 μl of ethyl acetate was added to the residue. The ethyl acetate solution was stored in a freezer at -20 °C until assayed. Samples of 3, 1, or 0.5 μl of this solution were introduced into the GCMS.

The following flowchart (Fig. II-5) shows biochemical techniques used in this study and the interrelationship of the three chromatographic analyses.

Figure II-5. Mutual relationship of chromatographic analyses. Three chromatographic techniques were employed to identify an endogenous catecholamines in *P. penicillatus*. Either homogenates or alumina extracts of tissue samples were introduced into either HPLC or TLC. The alumina extracts were introduced into GCMS to confirm the HPLC results. After a homogenate sample was separated on and extracted from TLC, it was introduced into the HPLC.


SAMPLE
Homogenization
Perchloric acid
HOMOGENATE
Alumina
ALUMINA-EXTRACT
HPLC-ED
TLC
Derivatization
Trifluoroacetate
GC-MS

RESULTS

A. Identification of Biogenic Amines from *Polyorchis penicillatus* with HPLC-ED.

Liquid chromatograms of standard amines, acidic metabolites and alumina extracts of tissue samples from HPLC system-I and -II are illustrated in Figs. II-6 and -7, respectively. By exploiting the hydrophobic interactions, I have separated a mixture of the amines and some metabolites in a single chromatographic run, using isocratic elution with an aqueous phosphate buffer. The extent of retention of catecholamines and their metabolites on a reversed-phase column is affected significantly by the pH of the eluent. At low pH values (< 3), the amino groups are fully protonated while dissociation of the carboxyl group is suppressed. The effects of pH on the retention times of amines and their acidic metabolites are shown in Fig. II-8. It is evident that suppressing the degree of dissociation increases solute hydrophobicity, and thus enhances the interaction between the solute molecules and the hydrocarbon stationary phase. Thus, the acidic metabolites are retarded longer at lower pH values, while the opposite is true for the catecholamines.

Figure II-6B demonstrates the presence, in the nerve-rich tissue sample, of small quantities of a dopamine-like compound and larger quantities of an norepinephrine-like compound having a retention time quite similar to that of authentic norepinephrine (12.13 min). However, identification and quantification of norepinephrine from such chromatograms were very difficult because of interfering compounds. Additional extraction methods, such as cation exchange to isolate amines or a solvent extraction, might be used to remove the interfering compounds. Unfortunately, there are serious disadvantages to this approach. Complex extraction procedures reduce the recovered amount of amines and overall sensitivity. Also it is difficult to use a single internal standard, and precision is reduced. Changes in the pH of the mobile phase over the range 2 to 5 did not significantly alter the retention time of norepinephrine in LC system-I (Fig. II-8). Therefore, to confirm the identification of both norepinephrine and dopamine in the sample, I used a second system (LC system-II) in which the constituents

Figure II-6. HPLC chromatograms (System I) of various amine standards, DOPA, and their metabolites compared with those from nerve-rich tissue.

A. A chromatogram of standard amines, DOPA, their metabolites and DHBA. It shows the separation obtained using the conditions of system-I (See materials and method) when 5 pmol of each amine standard and 5 pmol of DHBA were injected.

B. A chromatogram obtained when 30 µl samples of alumina extracted, nerve-rich tissue of *P. penicillatus* were injected. It is an example of a chromatogram obtained using system-I. It shows the presence of dopamine and a large, unidentified peak with a retention time similar to norepinephrine.

DA, dopamine; DHBA, 3,4-dihydroxybenzylamine; DOPA, 3,4-dihydroxyphenylalanine; DOPAC, 3,4-dihydroxyphenylacetic acid; EN, epinephrine; HIA, 5-hydroxyindoleacetic acid; 5HT, 5-hydroxytryptamine; HVA, homovanillic acid; NE, norepinephrine; ?, unidentified peaks.

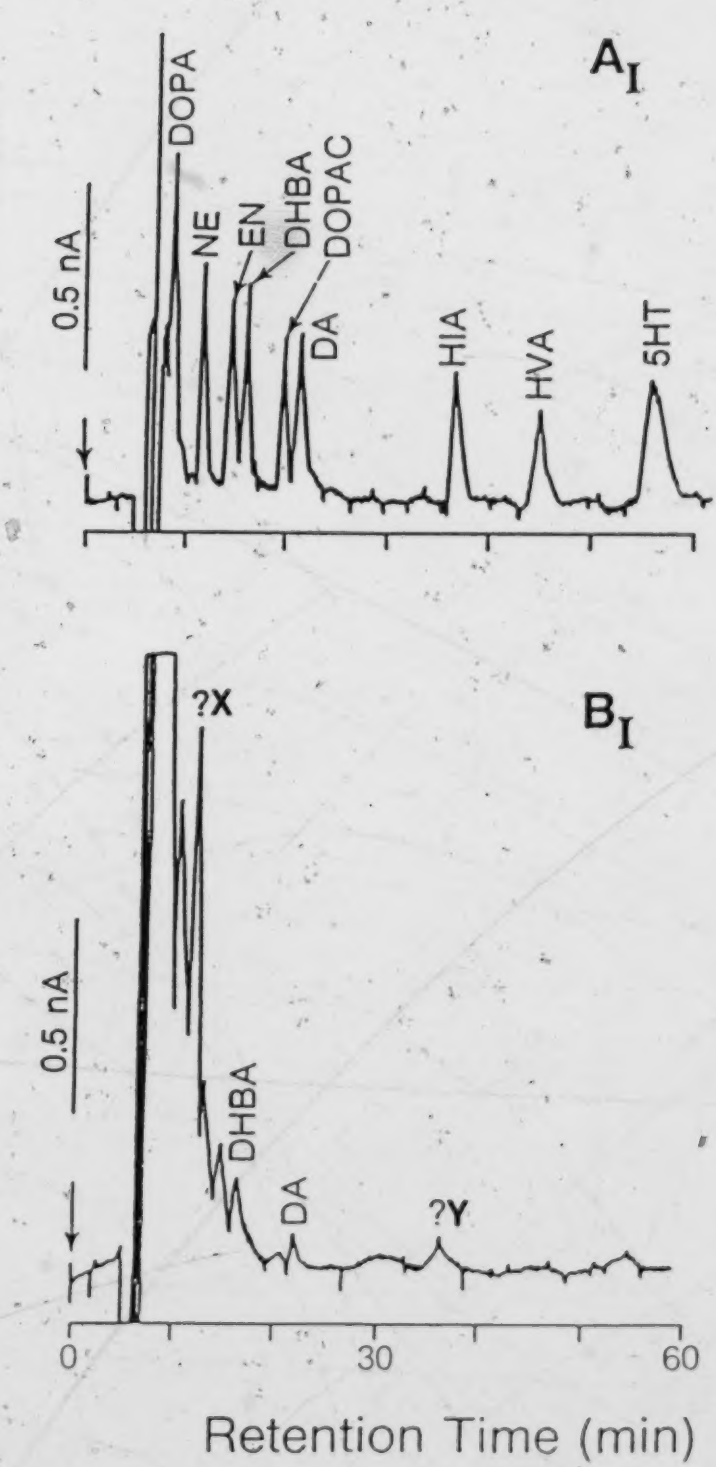

A_I
0.5 nA
DOPA
NE
EN
DHBA
DOPAC
DA
HIA
HVA
5HT
B_I
0.5 nA
?X
DHBA
DA
?Y
0
30
60
Retention Time (min)

Figure II-7. HPLC chromatograms (System II) of various amine standards, DOPA, and their metabolites compared with those from nerve-rich tissue.

A. A chromatogram of standard amines, DOPA, their metabolites and DHBA. It shows the separation obtained using the conditions of system-II (See materials and method) when 5 pmol of each amine standard and 10 pmol of DHBA were injected.

B. A chromatogram obtained when 30 µl samples of alumina extracted, nerve-rich tissue of *P. penicillatus* were injected. It is an example of a chromatogram obtained using system-II. It shows the presence of dopamine, but no peak is present having the same retention time as norepinephrine. The retention time of an unidentified compound (?X) was clearly different from that of norepinephrine. Abbreviations as for figure II-6.

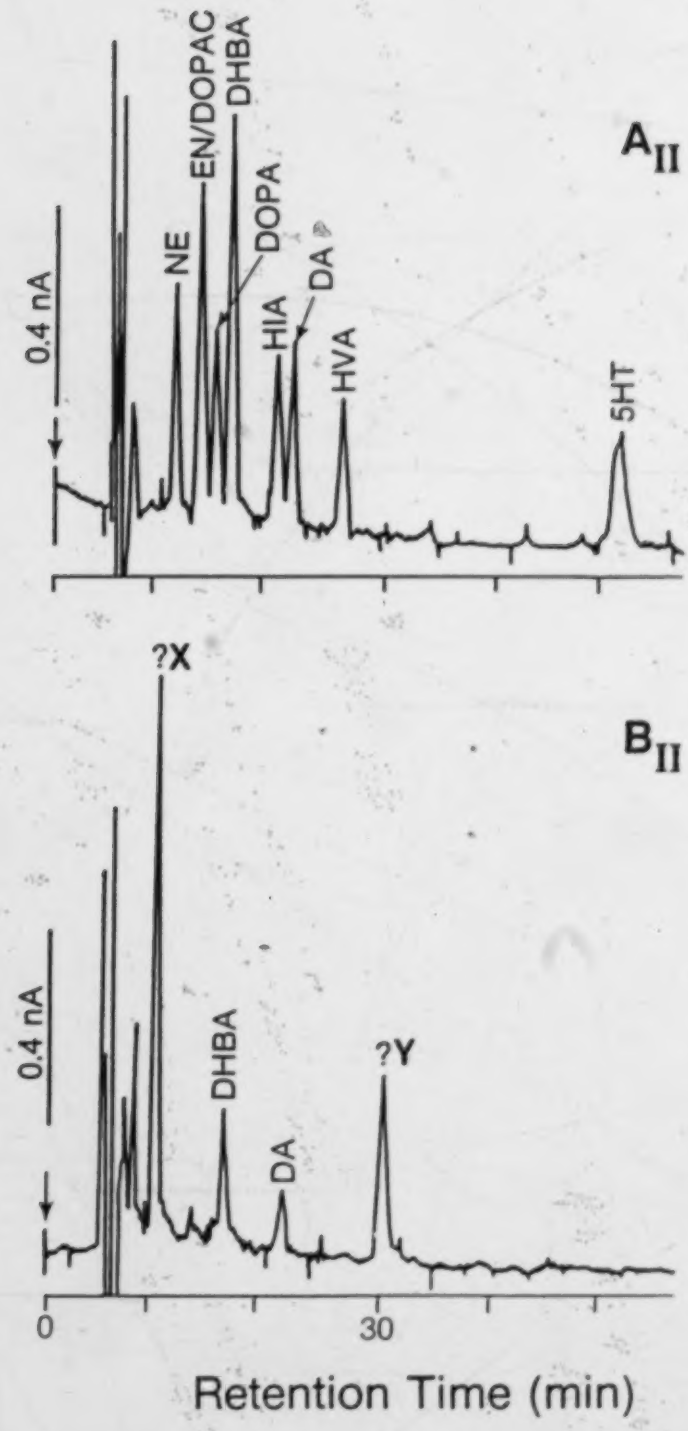
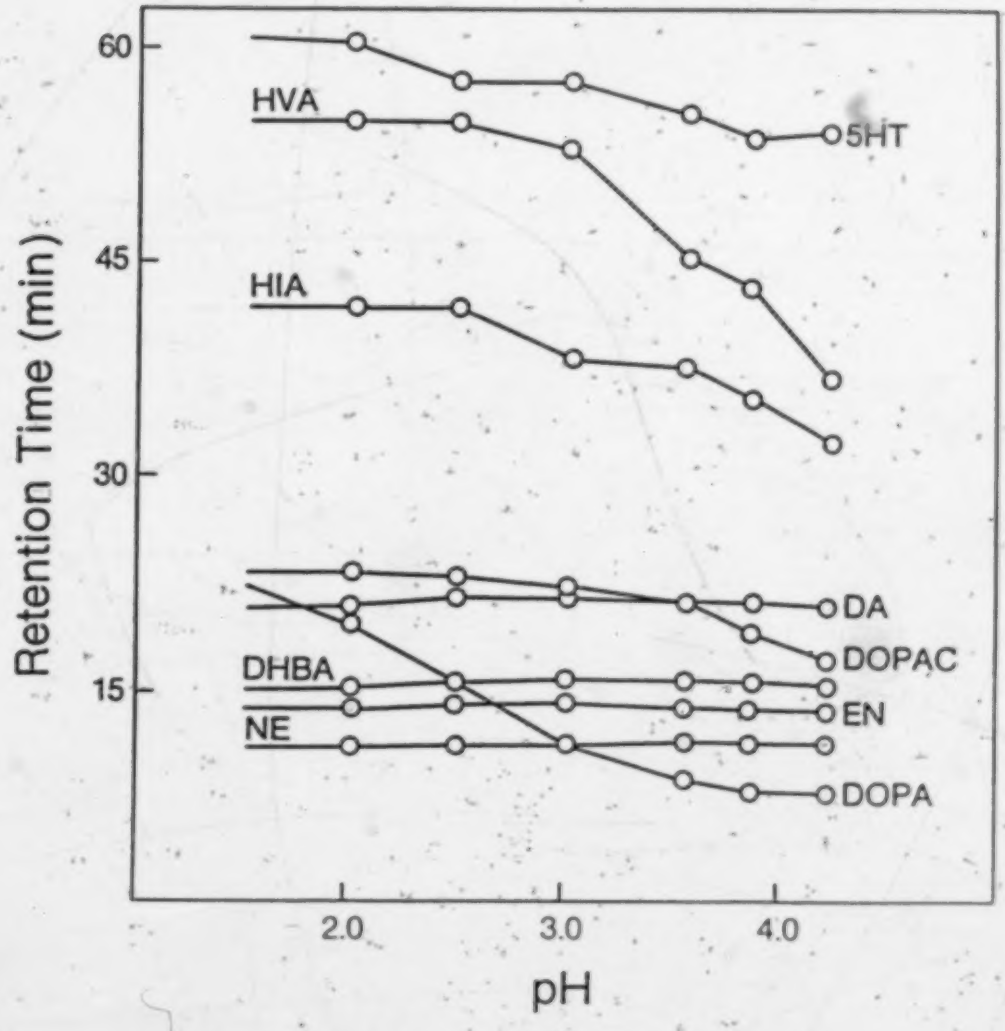

A II
0.4 nA
NE
EN/DOPAC
DHBA
DOPA
DA
HIA
HVA
5HT
B II
?X
0.4 nA
DHBA
DA
?Y
0
30
Retention Time (min)

Figure II-8. The effect of pH on the retention times of various amines, DOPA, their metebolites, and DHBA.

The graph was constructed using the mean retention times from at least 3 chromatograms at each of 6 pH values. The conditions for separation are those of system-I (See materials and method). Abbreviations as for figure II-6.


Retention Time (min)
60
45
30
15
HVA
5HT
HIA
DA
DOPAC
DHBA
EN
NE
DOPA
2.0
3.0
4.0
pH

of the mobile phase and the column itself were different from those of LC system-I. In system-II, the retention times of amines and metabolites were significantly different from those in system-I (Table II-2). Figure II-7B demonstrates the presence of dopamine and unknown compounds, one of which has been clearly retarded (8.35 min) less than authentic norepinephrine (10.86 min). The chromatogram obtained from homogenates rarely showed peaks corresponding to DOPA and DOPAC. However, the concentrations of either DOPA- or DOPAC-like compounds were so capriciously variable, from sample to sample, that I could not quantitatively determine their levels in tissue. Two unidentified peaks (compounds X and Y) were obtained from both alumina extracts and homogenates. These compounds appeared to have different retention times from those of any standard compounds in Table II-2. The fact that dopamine is present while norepinephrine is probably absent or at extremely low concentrations in tissue samples of *P. penicillatus* is further substantiated by the GCMS results.

Recovery of catecholamines from alumina extraction.

Catecholamines were reported (Anton & Sayre, 1962) to be apparently destroyed by perchloric acid when acid eluates were evaporated to dryness. Some of the alumina extracts used in this study had to be evaporated for GCMS experiments. Therefore, 0.1 N HCl was usually used for final elution instead of perchloric acid. The efficiency of the alumina extraction procedure was examined by comparing alumina-extracted and unextracted samples of known quantities of catecholamines (Table II-3). Recovery was calculated assuming that the volume of the alumina eluate equals the volume of acid added and was thus not corrected for the dilution caused by water remaining on the alumina after the final wash (ca. 100 µl). Table II-4 shows that there is no significant change in retention times of catecholamines after alumina extraction.

Matrix effect.

The homogenate was divided into two equal portions. Known quantities of standard substances and internal standards were added to one tube (A), while only internal standards were added to another (B). The same amounts of the standard substances were added to tubes (C) without tissue in order to determine the effect of tissue on the

Table II-2. Retention times of various amines and metabolites on two different high performance liquid chromatographic systems.

	System I	*System II*
1. NE	12.13 ± 0.20	10.86 ± 0.04
2. EN	15.45 ± 0.21	13.13 ± 0.15
3. DHBA	17.02 ± 0.17	16.05 ± 0.05
4. DA	23.38 ± 0.20	21.47 ± 0.29
5. EPININE	*	25.41 ± 0.13
6. 5HT	53.40 ± 0.28	50.48 ± 0.12
7. DHPG	*	7.05 ± 0.13
8. DOPA	7.80 ± 0.20	14.45 ± 0.07
9. DOPAC	18.80 ± 0.15	12.95 ± 0.06
10. HIA	35.40 ± 0.23	20.02 ± 0.05
11. HVA	43.20 ± 0.31	26.09 ± 0.10

Values are expressed in minutes (mean ± S.D. ; N = 10).
* NOT RUN

Table II-3. Recovery of catecholamines and DHBA as a function of alumina extraction.

NE	*EN*	*DHBA*	*DA*
Extracted standard (N=10)			
0.69±0.02	0.62±0.04	0.74±0.04	0.51±0.05
Unextracted standard (N=10)			
1.04±0.02	0.88±0.02	0.94±0.02	0.71±0.01
% recovery			
66.4±0.6	70.5±2.9	78.7±2.5	71.8±6.5
% recovery relative to DHBA			
84.3±1.9	89.5±0.8	100	91.3±5.1

Values are given in mean peak response (nA ± S.D.).

Table II-4. Retention times of catecholamines after alumina extraction.

NE	*EN*	*DHBA*	*DA*
Unextracted standard (N=10)			
12.13±0.20	15.45±0.21	17.02±0.17	23.38±0.20
Extracted standard (N=10)			
12.13±0.09	15.38±0.11	16.92±0.15	23.32±0.08
Extracted standard with tissue (N=5)			
12.25±0.25	15.33±0.22	16.89±0.13	23.40±0.21

Values are given in min and obtained using LC-system I.

recovery of catecholamines (matrix effect). The possible endogenous amine content in tissue homogenates was subtracted from the quantities of standard amines added exogenously to tissue homogenates by comparing the chromatographic results from sample A with those of sample B. The recovery ratios shown in Table II-5 were calculated by comparing the peak height of each substances in a sample having tissue (A-B) with that lacking tissue (C). Mesoglea did not produce a significant change in the recovery efficiency of catecholamines. However, the recovery of either norepinephrine or dopamine from alumina extraction was affected more or less by other tissues; 92% of dopamine was recovered from alumina extraction in the presence of nerve-rich tissue. The reciprocals of the percentage recoveries were considered as correction factors due to matrix effect, K, to determine the concentrations of dopamine in several different tissues.

Recoveries of catecholamines relative to that of DHBA were not significantly affected by the sample matrix, thus justifying the use of DHBA as an internal standard for this experiment (see Appendix 3).

B. Demonstration of the Presence of Biogenic Amines using TLC.

Five clearly separated fluorescent bands were observed on the chromatography plates using standard catecholamines, DOPA and serotonin, after elution with butanol-acetic acid-water (Fig. II-9). The white-blue fluorescence of four of the bands corresponded to DOPA and catecholamines while the red fluorescence of the fifth band corresponded to serotonin. Para- and meta-octopamine and the acidic metabolites of amines such as DOPAC, HVA, DHPG, and HIAA were clearly separated and distinguished from catecholamines and serotonin. Their R_f values were distinctly greater than those of the catecholamines and serotonin and these compounds did not produce any fluorescence after reacting with paraformaldehyde. The low sensitivity of the detection method used here may be one of the reasons that octopamine at 10 nmol, both para- and meta-form, did not produce fluorescence under UV light after reacting with 4% paraformaldehyde solution. The tissue samples increased the developing time of amines on the plates but had no effect on their R_f values. The values of R_f x 100 are

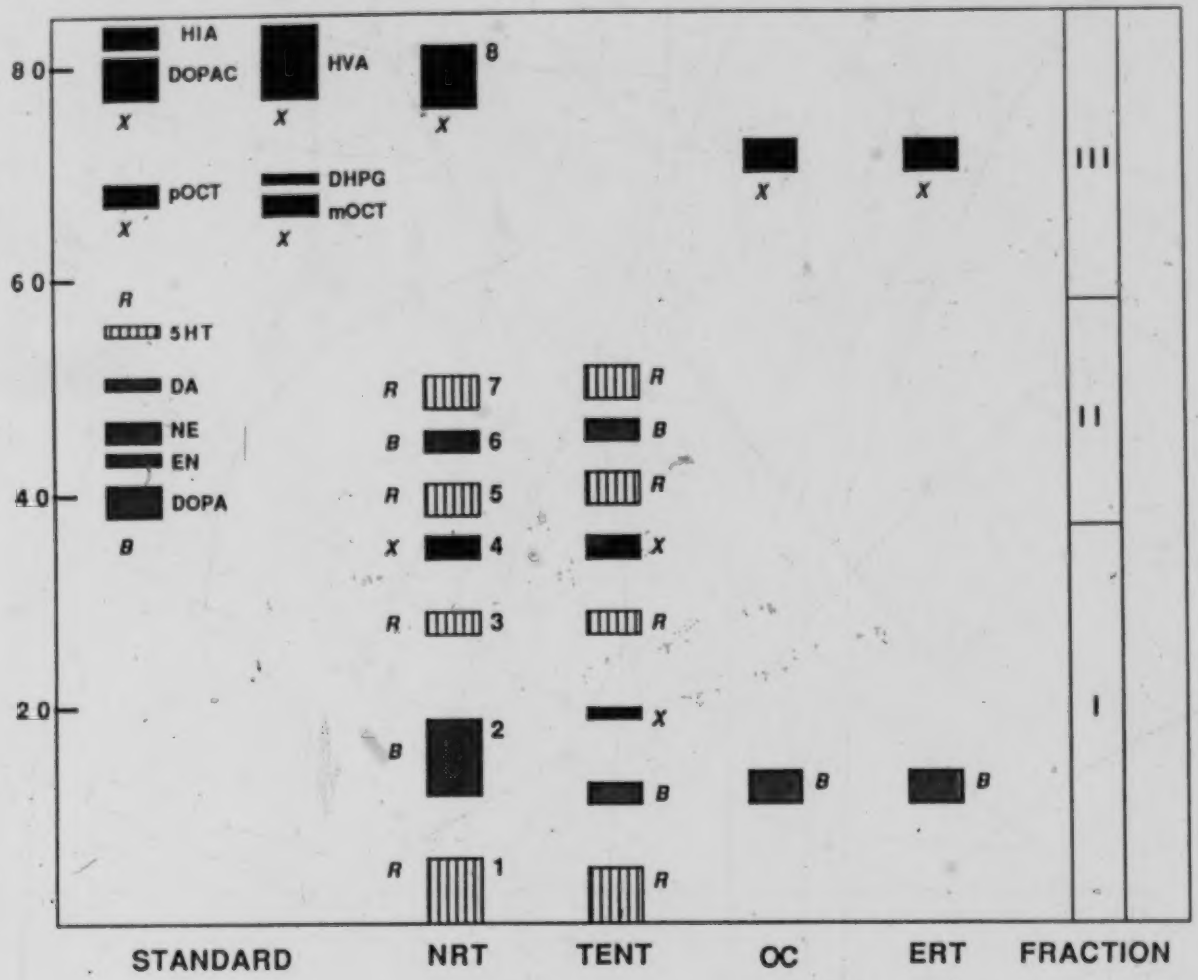
Table II-5. Percentage recovery of catecholamines and DHBA in the presence of tissue (matrix effect).

NE	*EN*	*DHBA*	*DA*
Nerve-rich tissue (N=5)			
90.1±7.8	98.8±2.0	98.7±5.1	92.4±0.9
Endoderm-rich tissue (N=5)			
91.1±5.4	100.9±1.8	92.5±1.5	102.3±3.9
Mesoglea (N=3)			
100.6±6.0	99.4±6.6	98.4±2.9	99.0±3.8

Values are expressed as % (mean± S.D.).
Each sample was prepared from 5 to 8 jellyfish.

Figure II-9. Thin layer chromatogram of standard amines, their metabolites and dried tissue samples from *P. penicillatus*.

Each of 10 µl of 1 mM standard catecholamines and DOPA solutions (10 nmoles in 0.1 N perchloric acid) was applied to a TLC plate. They were clearly separated on the TLC plate and generated their characteristic fluorescence when they reacted with aldehyde: Whitish-blue fluorescence (***B***) from DOPA and catecholamines and red (***R***) from serotonin (5HT). Octopamine, acidic metabolites of monoamines and DHPG did not produce any fluorescence. However, they were easily spotted on a TLC plate since they remained on the plate as dark brown scorch marks (***X***) after baking in an oven. Homogenates (in 0.1 N perchloric acid) of dried tissue samples were used in TLC analysis. Several fluorescent bands appeared from tissue samples, especially from NRT and TENT. Their R_f values and fluorescent colours (i.e., excitation wavelength) did not match those of standard compounds. Therefore, the compounds in the NRT, producing fluorescent bands 5, 6 and 7, are unlikely to be catecholamines. Fraction II which was supposed to include these compounds was introduced into HPLC system II (see the following figure, II-10). The R_f value of band 8 appearing in only NRT was similar to that of DOPAC. [a DOPAC-like compound appeared in the HPLC chromatogram for Fraction III which was supposed to include a compound corresponding to band 8. See figure II-10.] *NRT, nerve-rich tissue; TENT, tentacle; OC, ocelli; ERT, endoderm-rich tissue.*

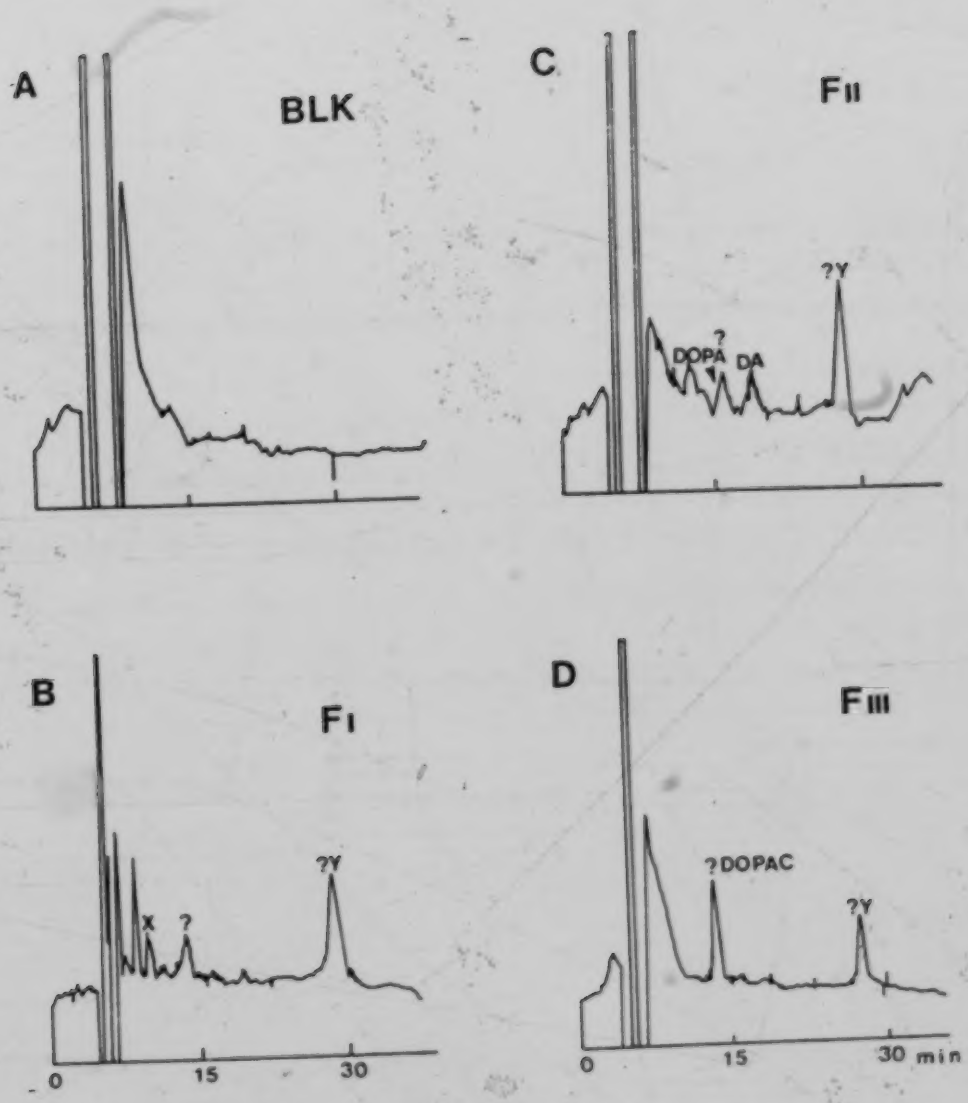

80
60
40
20
HIA
DOPAC
HVA
8
pOCT
DHPG
mOCT
5HT
DA
NE
EN
DOPA
7
6
5
4
3
2
1
III
II
I
STANDARD
NRT
TENT
OC
ERT
FRACTION

presented in Fig. II-9. Each of 0.05 g of dried nerve-rich tissues and tentacles produced TLC chromatograms having 8 bands, while the same amount of ocelli and radial canal tissue produced chromatograms having only two bands (Fig. II-9). The yellow fluorescence of band 8 in the nerve-rich tissue sample had a high R_f value. This, however, gave little information as to whether or not the compound of band 8 corresponded to the catechol-4, with a high R_f value on a paper chromatogram, which Östlund (1954) identified. Fluorescent bands 5, 6, 7 (red, blue and red, respectively) of nerve-rich tissue appeared on regions of the plate where standard monoamines had located but their R_f values and colors were different from those of standard amines. Furthermore, all these fluorescent bands were not observed after extracting with alumina. Approximately 0.05 g of dry sample of nerve-rich tissue corresponded to around 0.45 g of wet weight since the ratio of dry samples to wet was approxiamtely 9 (refer to the Fig. II-1). Thus, dry samples of nerve-rich tissues used in this experiment should contain less than one nanomole of dopamine (refer to the figure II-17 showing dopamine content in nerve-rich tissue) which is below the detection level of the method used. When larger quantities of dry sample were used, the fluorescent bands on the plate overlapped and could not be distinguished from one another.

I ran an HPLC analysis on the TLC fractions I, II and III (Fig. II-9) in order to; 1) determine whether the dried nerve-rich tissue contained catecholamines, 2) confirm the previous HPLC observations that neither norepinephrine nor epinephrine were present in the nerve-rich tissue and that only dopamine was present, 3) determine the R_f values of TLC for the unknown compounds X and Y appearing on the LC chromatograms. This was not entirely satisfactory because of the presence of the unknown peaks from the TLC fractions appearing on the LC chromatograms (Fig. II-10) and the extremely low recovery of amines from silica gel when acidic methanol was used as the extracting solvent (less than 5% yield in the case of dopamine). These results (Fig. II-9), however, suggest the following; 1) DOPA and dopamine are present in Fraction II and correspond to the catecholamine region of the TLC, 2) norepinephrine and epinephrine are absent from the nerve-rich tissue, 3) the unknown compound X is present in the TLC Fraction I. Because both catechol-4 and the unknown compound X have low R_f values on open chromatography, i.e. paper and thin layer chromatography

Figure II-10. Liquid chromatograms of TLC fractions of nerve-rich tissue of *Polyorchis*.

The acidic eluate of each fraction from the TLC plate (refer to the previous figure, II-9) was introduced to LC system-II. A shows a blank chromatogram (BLK) for an acidic eluate of the TLC plate itself. The other panels, B, C and D show respectively the chromatograms for acidic eluates of the TLC fractions I, II and III of nerve-rich tissue.


A
BLK
C
FII
?Y
DOPA
?
DA
B
FI
?Y
X
?
0
15
30
D
FIII
?DOPAC
?Y
0
15
30 min

respectively, and probably are present in fairly large amounts in the tissues used, the unknown compound X may be catechol-4, 4) the unknown compound Y appears in every fraction (Figs. II-9 & -10).

C. GCMS Analysis.

Several standard catecholamines, DOPA and DHBA were derivatized with TFAA in ethyl acetate and introduced into the GCMS in the negative ion chemical ionization (NICI) mode where methane was used as the reagent gas. The resultant total ion current chromatogram is shown in Fig. II-11, the mass spectra of TFA-derivatized standards are shown in Fig. II-12, and the retention times and major ion intensities of these compounds are listed in Table II-6. In the NICI mode, saturation of the ion signals occurred with sample quantities as small as 500 pmole per injection. There was a sudden decrease in total ion current (TIC) using samples with less than 1.0 pmole per injection and this seemed to be occurring in the GC stage rather than in the MS. This was determined by injecting 1.0 pmole of authentic dopamine derivative in the ion source using a direct insertion probe, which caused the selected ion current to be saturated. A 100-fold increase in the sensitivity of the selective ion current was observed when the MS was set for the selective ion monitoring (SIM) mode. However, this mode was rarely used in this study because the amount of sample obtained by derivatization was too small to be used repeatedly. In NICI mass spectra, a peak of $(M + 17)^-$ often appeared, which was probably due to OH^- clusters when the methane pressure of the ion source was high or the sample was particularly impure. One µl of derivatized alumina extract was injected to the GC injector in the splitless mode in order to obtain the TIC chromatograms of a real sample. The amount of derivatized dopamine in the 1.0 µl of injected fluid was approximately 8 pmoles. This value was determined by quantifying 30 µl of alumina extract with the HPLC-ED before drying and derivatization assuming 100% derivatization yield and no handling loss. The recovery yield of catecholamines determined by HPLC was approximately 100% at the drying step under nitrogen as well as air. Interestingly, the recovery yield of serotonin after the drying step was extremely low, almost zero, under a stream of air.

Figure II-11. Total ion current chromatogram of the TFA-derivatives of standard catecholamines, DOPA and DHBA.

Fifty pmoles of each amine standard were introduced into the GCMS.

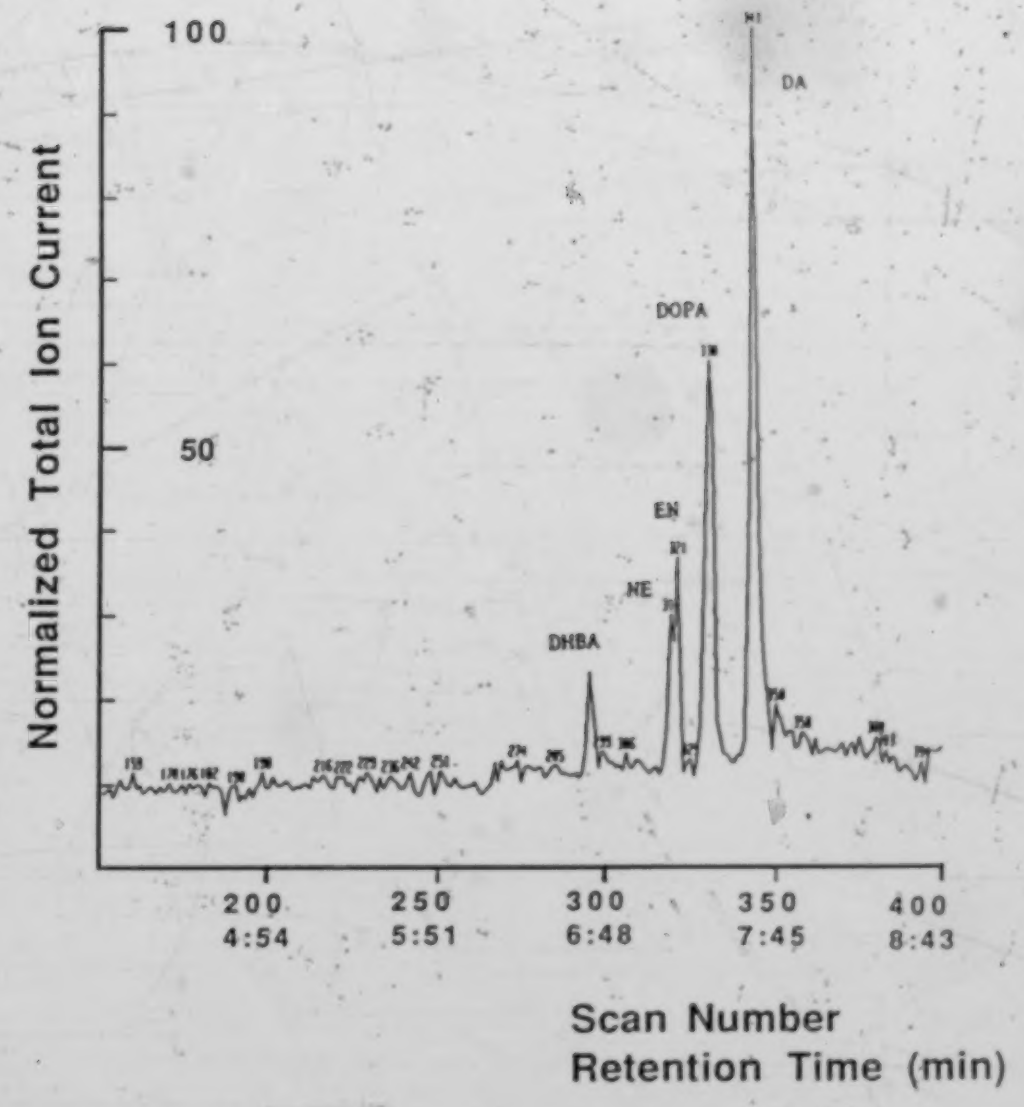

100
50
Normalized Total Ion Current
DA
DOPA
EN
NE
DHBA
200
4:54
250
5:51
300
6:48
350
7:45
400
8:43
Scan Number
Retention Time (min)

Figure II-12. Negative ion chemical ionization mass spectra (NICI-MS) of standards.

Typical spectra showing the characteristic fragmentation patterns of the TFA derivatives of the amine standards, DHBA, and DOPA. For example, dopamine produces a molecular ion with a mass of 441 and fragment ions having masses of 344 and 248. Conditions for producing these spectra are described in materials and methods.

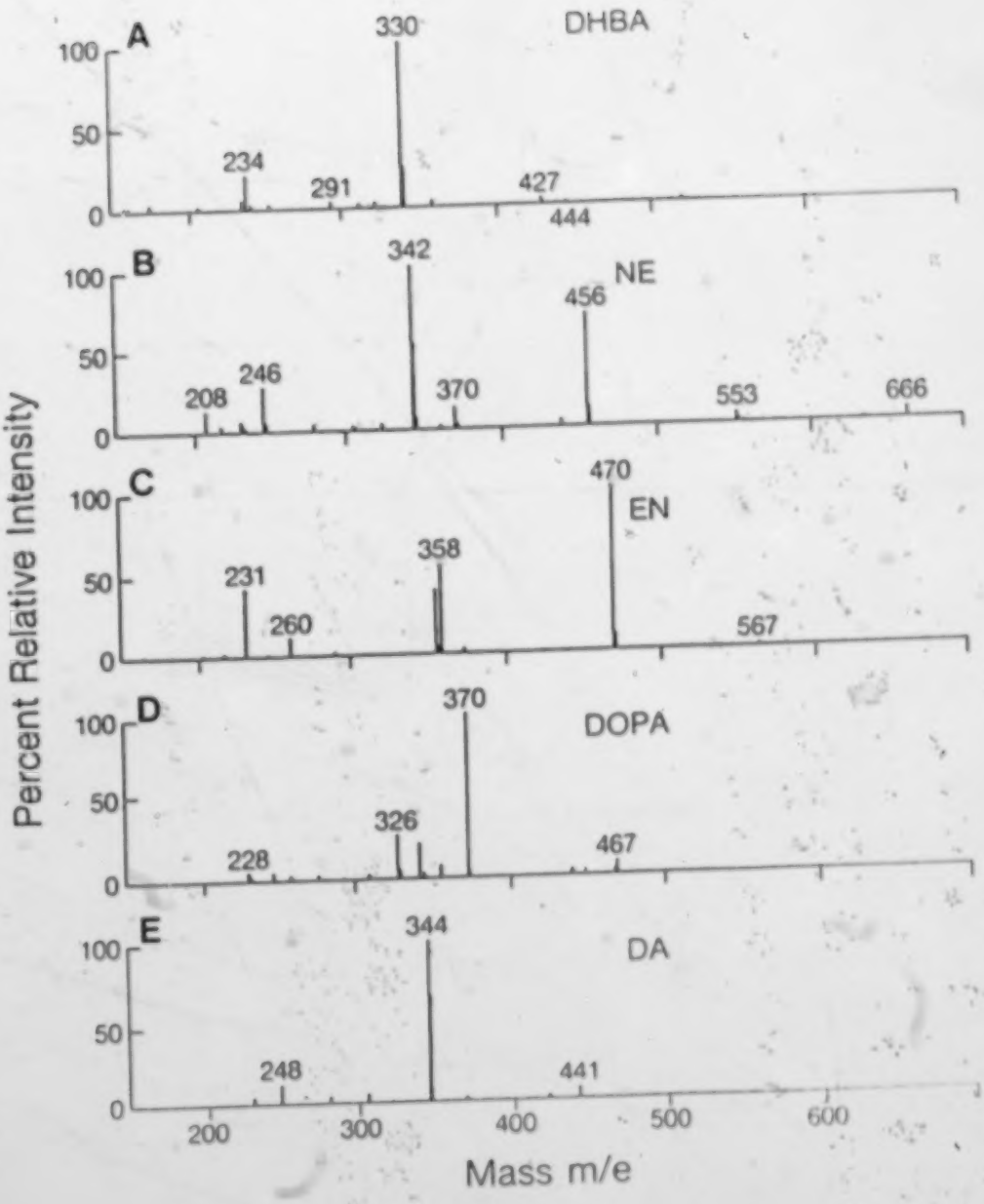

A
DHBA
330
234
291
427
444
B
NE
342
456
246
208
370
553
666
C
EN
470
358
231
260
567
D
DOPA
370
326
228
467
E
DA
344
248
441
100
50
0
200
300
400
500
600
Percent Relative Intensity
Mass m/e

Table II-6. NICI-GC/MS characteristics of the TFA derivatives of biogenic amines.

TFA Derivative	Retention times	Molecular ion (m/e)	Relative intensity of peaks (%) M⁻	[M-TFA]⁻	[M-TFA-TFAOH]⁻
Standard					
DHBA	6:37	427	3.8	100	0
NE	7:05	553	10.7	67.4	100
EN	7:07	567	1.6	100	39.0
DOPA	7:17	467	8.5	100	3.0
DA	7:32	441	7.5	100	0
Sample					
DHBA	6:51	427	4.2	100	0
DA	7:45	441	4.3	100	0

Values of retention times are expressed in min:sec.

TFA, trifluoroacetyl group (CF_3CO^-); TFAOH, trifluoroacetic acid (CF_3COOH).

Figure II-13. Chromatograms of alumina-extracted, nerve-rich tissue from *Polyorchis penicillatus* using NICI GC/MS.

(A). Total ion current chromatogram from scan number 90 to 350 showing dopamine at scan number 297 with a retention time of 7 min 45 sec. Besides the internal standard, DHBA, there are numerous unidentified peaks, some of which are due to TRIS and EDTA.

(B). Selected ion current chromatogram for one of the characteristic fragment ions of dopamine (m/e = 344) showing that the peak occurs at the scan number 297.

(C). Selected ion current chromatogram for the characteristic molecular ion of dopamine (m/e = 441) showing that the peak occurs at the scan number 297.

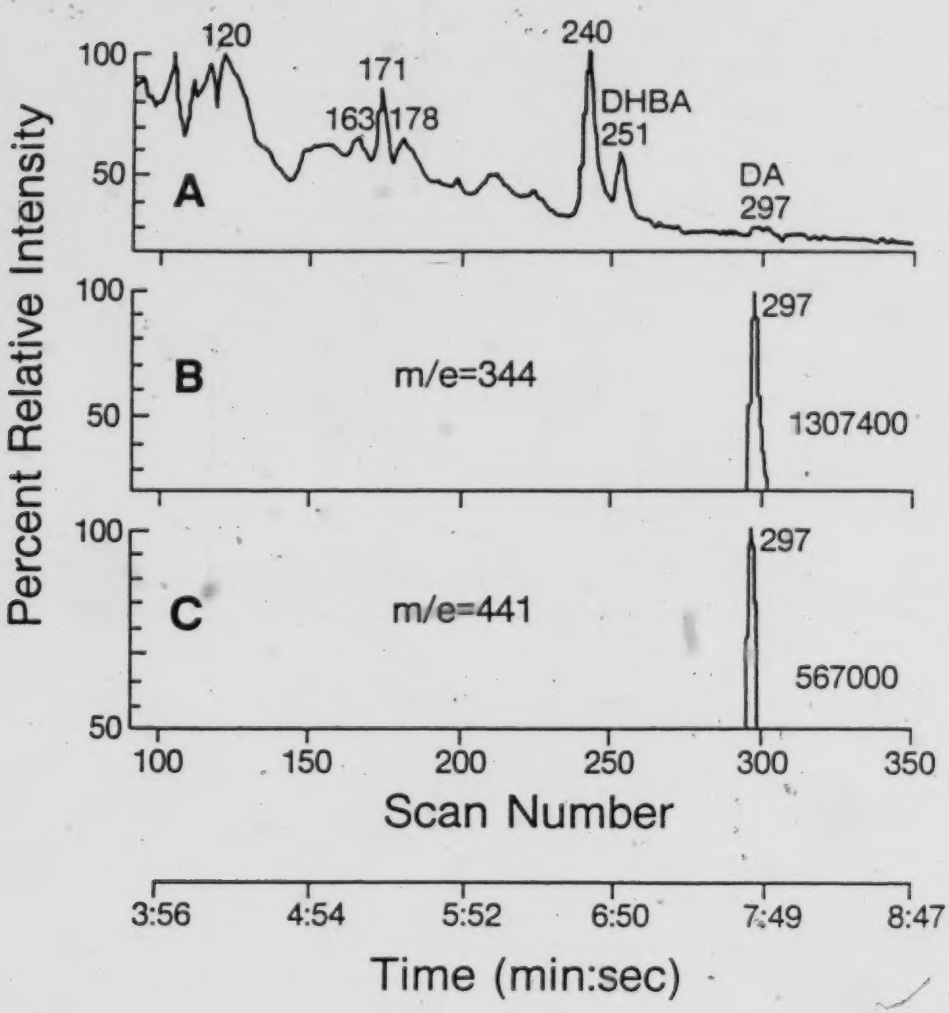

Percent Relative Intensity
100
50
A
120
171
163
178
240
DHBA
251
DA
297
B
m/e=344
297
1307400
C
m/e=441
297
567000
100
150
200
250
300
350
Scan Number
3:56
4:54
5:52
6:50
7:49
8:47
Time (min:sec)

Figure II-13A shows the total ion current (TIC) chromatogram of TFA-derivatized samples. The large tailing peaks up to scan number 120 come from TFA-derivatives of TRIS residues that were used in the alumina extraction procedure. The NICI chromatogram of the TRIS derivatives is shown in Fig. II-14 and their mass spectra are shown in Fig. II-15. Two major products would be formed from the reaction of TRIS with TFAA: one with a molecular weight of 601 and another of 505. However, there were no such molecular anions for TRIS, as seen from the spectra. Instead, there were ions at 618 and 522 which corresponded to the OH^- adducts of the respective molecules. Although the mass spectra of TRIS in the TIC of a nerve-rich tissue sample were saturated, there was no difficulty in identifying them because the major ions in the spectra of TFA derivatives of the authentic TRIS were present in spectra of nerve-rich tissue.

The molecular mass of derivatized dopamine is 441. The NICI spectrum of dopamine derivatives (Fig. II-12E) showed a peak where molecular weight, m/e, is 441 and a small peak at 458 which is the M + 17 ion, $(M + OH)^-$. The most reproducible and largest base peak corresponded to an ion of m/e=344 due to the loss of 97 (CF_3CO) from the molecular anion. To confirm the presence of dopamine derivatives in the GCMS spectrum of nerve-rich tissues of *Polyorchis penicillatus*, selected ion chromatograms (SIC) were reconstructed from the TIC chromatogram for the two informative ions of TFA-derivatized dopamine. These were the ions at m/e = 441, which is the molecular anion, and 344, the base peak. From these two SICs (Figs. II-13B & C), it can be seen that the peak at scan number 297 corresponds to TFA-derivatized dopamine. The intensity ratio of the two ions (441/344) in the chromatograms was 0.043 but the intensity ratio of standard dopamine was 0.075 (Table II-6). This variation is probably due to the inconsistency of the ion source pressure. There is a slight difference in the GC retention times of standard dopamine from that of the samples. The retention time of dopamine in a sample derivative was 7 min 45 sec while that of standard dopamine was 7 min 32 sec. The NICI spectrum of scan number 297 is shown in Fig. II-16. In the spectrum, an ion at m/e = 458 which corresponds to $(M + 17)^-$ did not appear. But when 3 µl instead of 1 µl of sample solution was introduced into the GC injector, the ion at m/e = 458, whose intensity was twice as high as that of the

Figure II-14. Total ion current chromatogram of the TFA derivatives of TRIS.

Alumina extracts were found to contain TRIS molecules. However, these TRIS molecules did not interfere with GCMS analysis for catecholamines. (Compare with figure II-11).

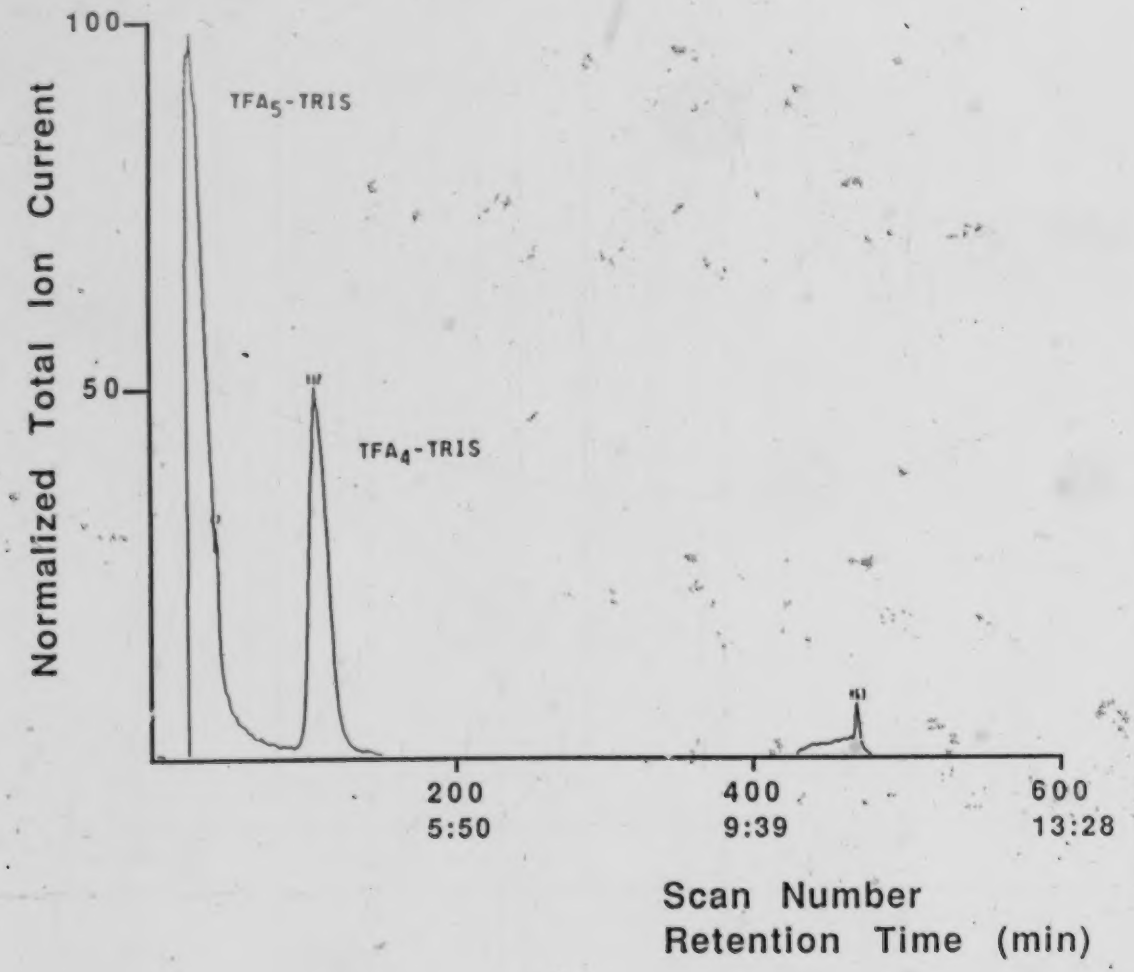

100
50
Normalized Total Ion Current
TFA5-TRIS
TFA4-TRIS
200
5:50
400
9:39
600
13:28
Scan Number
Retention Time (min)

Figure II-15. NICI mass spectra of TFA derivatives of TRIS.

A. Mass spectrum obtained from a TFA_5-TRIS derivative.

B. Mass spectrum obtained from a TFA_4-TRIS derivative.

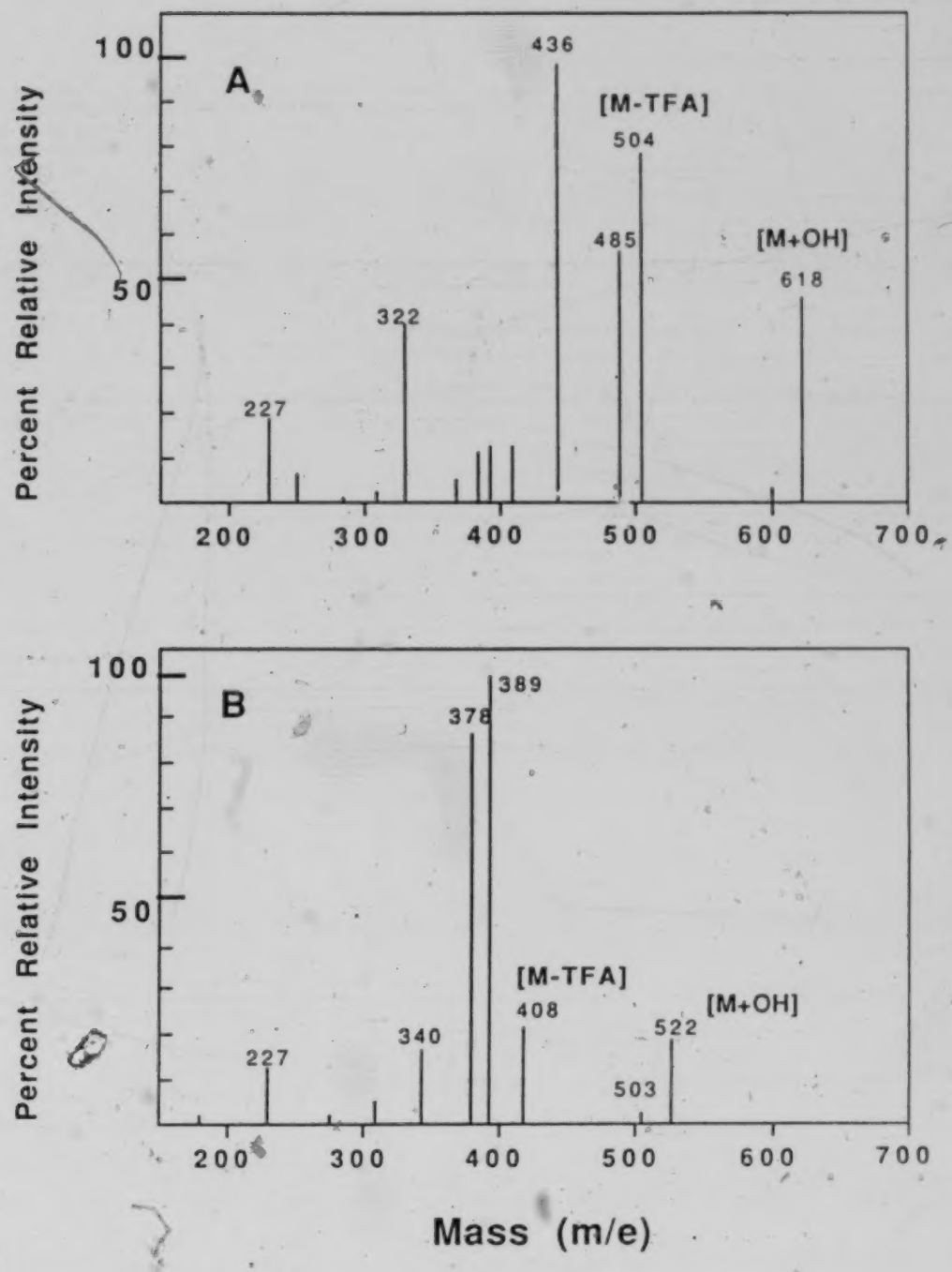
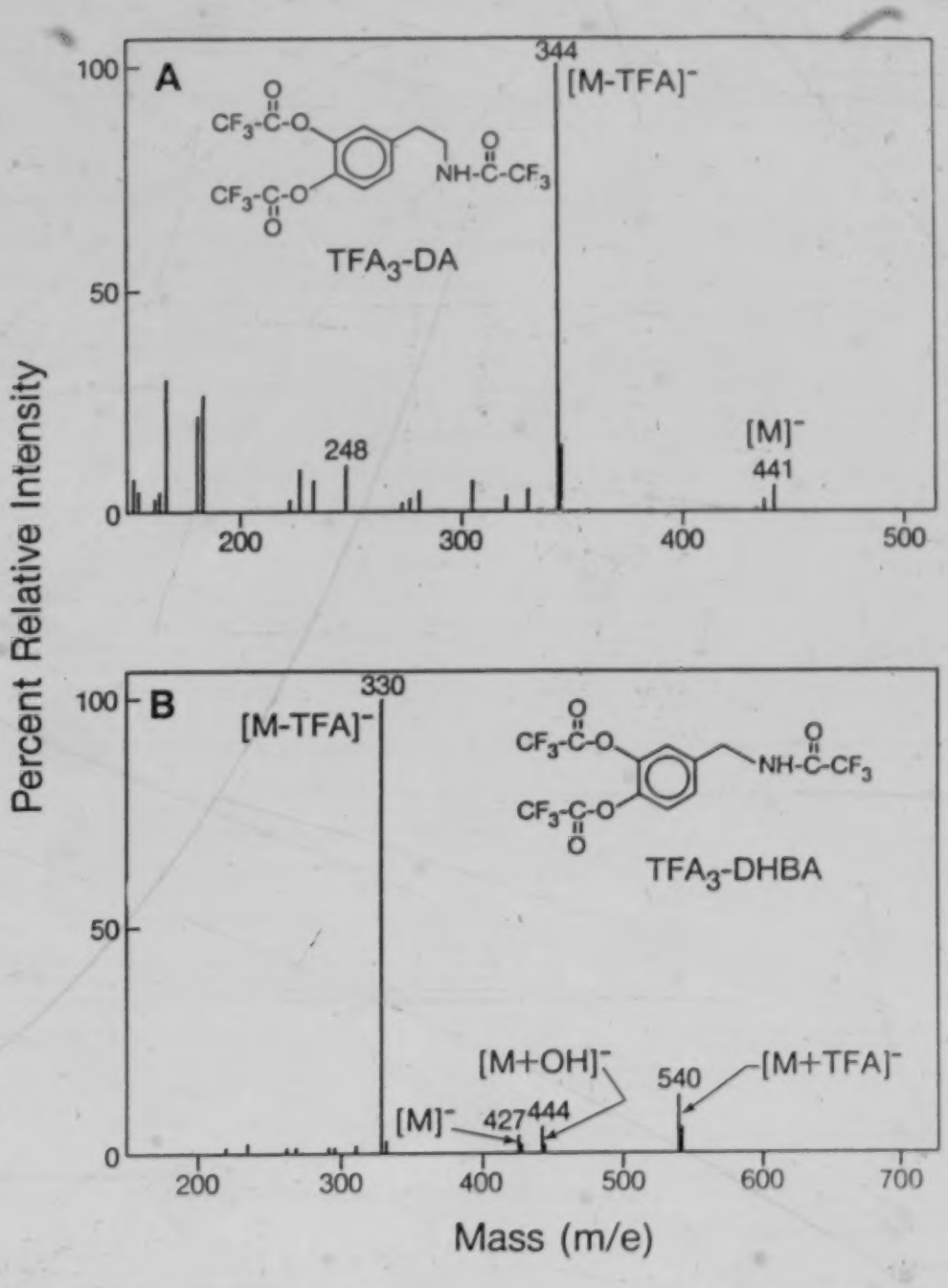

A
100
50
436
[M-TFA]
504
485
[M+OH]
618
322
227
Percent Relative Intensity
200
300
400
500
600
700
B
100
50
389
378
[M-TFA]
408
[M+OH]
522
340
227
503
Percent Relative Intensity
200
300
400
500
600
700
Mass (m/e)

Figure II-16. NICI Mass spectra showing the presence of dopamine in alumina-extracted, nerve rich tissue of *P. penicillatus*.

A. NICI spectrum of scan number 297 from the total ion current chromatogram of figure II-13-A showing the characteristic mass spectrum for TFA-derivatized dopamine; compare with figure II-12.

B. NICI spectrum of scan number 251 from the total ion current chromatogram of figure II-13A showing the characteristic mass spectrum of the internal standard, DHBA after derivatization with TFAA; compare with figure II-12.


A
344
[M-TFA]⁻
100
50
0
248
[M]⁻
441
200
300
400
500
TFA3-DA
B
330
[M-TFA]⁻
[M+OH]⁻
540
[M+TFA]⁻
[M]⁻
427
444
600
700
TFA3-DHBA
Percent Relative Intensity
Mass (m/e)

molecular anion, was observed. There were still other unmatched peaks between the two mass spectra. These peaks could be due to strong background ions because they were also observed in the spectra at other scan numbers. Comparison of the spectrum of scan number 297 on the TIC chromatogram of TFA-derivatized samples with that of TFA-derivatized dopamine standard (Fig. II-11E) shows that the peak at scan number 297 is due to the TFA-derivatized dopamine in the nerve-rich tissue.

The NICI spectrum of authentic norepinephrine derivative is shown in Fig. II-12B. The molecular mass of TFA-derivatized norepinephrine is 553. Ions at m/e=553 and m/e=660 appeared on the spectrum. The latter corresponds to M + 113 (M + CF_3CO_2). This peak is thought to be formed by an ion molecular reaction occurring in the ion source; this ion molecular reaction would occur if the concentration of the sample were high. When 8.5 pmoles of norepinephrine was introduced into the ion source in the split-less mode, no such peak appeared. To examine whether there is norepinephrine in nerve-rich tissues, SICs were reconstructed from the TIC chromatogram for characteristic ions of m/e = 553, 456, 439, 344, and 342. The samples did not give any peaks in the SICs corresponding to m/e = 553 and 342, and the SICs corresponding to m/e = 344 and 439 had different retention times from those of standard norepinephrine. However, in the SIC corresponding to m/e = 456, a peak (scan number 268 of the TIC chromatogram) appears having a similar retention time to standard norepinephrine. The mass spectrum of scan number 268 shows that the ion at m/e = 456 is within the noise of the entire spectrum. Thus, it is evident that if norepinephrine is present in the samples, it must occur at levels below the detection limit of the GCMS system used in this study, which is approximately 1 pmole per injection.

The SICs for several characteristic ions of epinephrine, DOPA and DHBA were examined. The ions of m/e= 567 $[M]^-$ and 470 $[M\text{-}TFA]^-$ for epinephrine were used for reconstructing chromatograms from the NICI spectrum and the ions of m/e= 467 $[M]^-$ and 370 $[M\text{-}97]^-$ for DOPA were used. Both SICs and mass spectra for epinephrine and DOPA could not be obtained from the TIC chromatogram of sample derivatives. It is probable from these results, combined with the previous HPLC and TLC results, that epinephrine is not present in *P. penicillatus*. However, this GCMS result does not

necessarily mean that DOPA is not present in the Hydrozoa, since 'alumina' extracts were used for the GCMS analysis. Both SICs of m/e = 330 and 427, which are characteristic ions of DHBA, corresponded to the peak of scan number 251 of the TIC chromatogram. The mass spectrum of scan number 251 coincided with that of standard DHBA, indicating that the peak of scan number 251 came from DHBA derivatives which were added as internal standards (Fig. II-168).

D. Distribution of Biogenic Amines in *Polyorchis penicillatus* as determined by HPLC.

Dopamine content in various tissues of Polyorchis.

The dissection technique used in this study did not permit isolation of purely neuronal samples in *P. penicillatus*., although nerve cells are primarily concentrated around the animal's bell margin. The nerve-rich tissues that were used in this study consisted of neurons, ectodermal cells, a small amount of mesoglea, and endodermal cells from the ring canal. Radial canal tissue was used as an example of endoderm-rich tissue. Figure II-17 shows the quantity of dopamine present in different tissues of *P. penicillatus*. Dopamine was present in the highest concentrations in nerve-rich tissue (120 fmol/mg wet wt), at intermediate concentrations in endoderm-rich tissue (30 fmol/mg wet wt.), and at the lowest concentrations in the mesoglea (10 fmol/mg wet wt.). Some of the dopamine detected in radial canal tissue could have been present in neurons of the radial nerves which are found in this tissue (Spencer, 1979). All the animals used in this study were starved for at least two days prior to dissection to avoid any exogenous sources of dopamine.

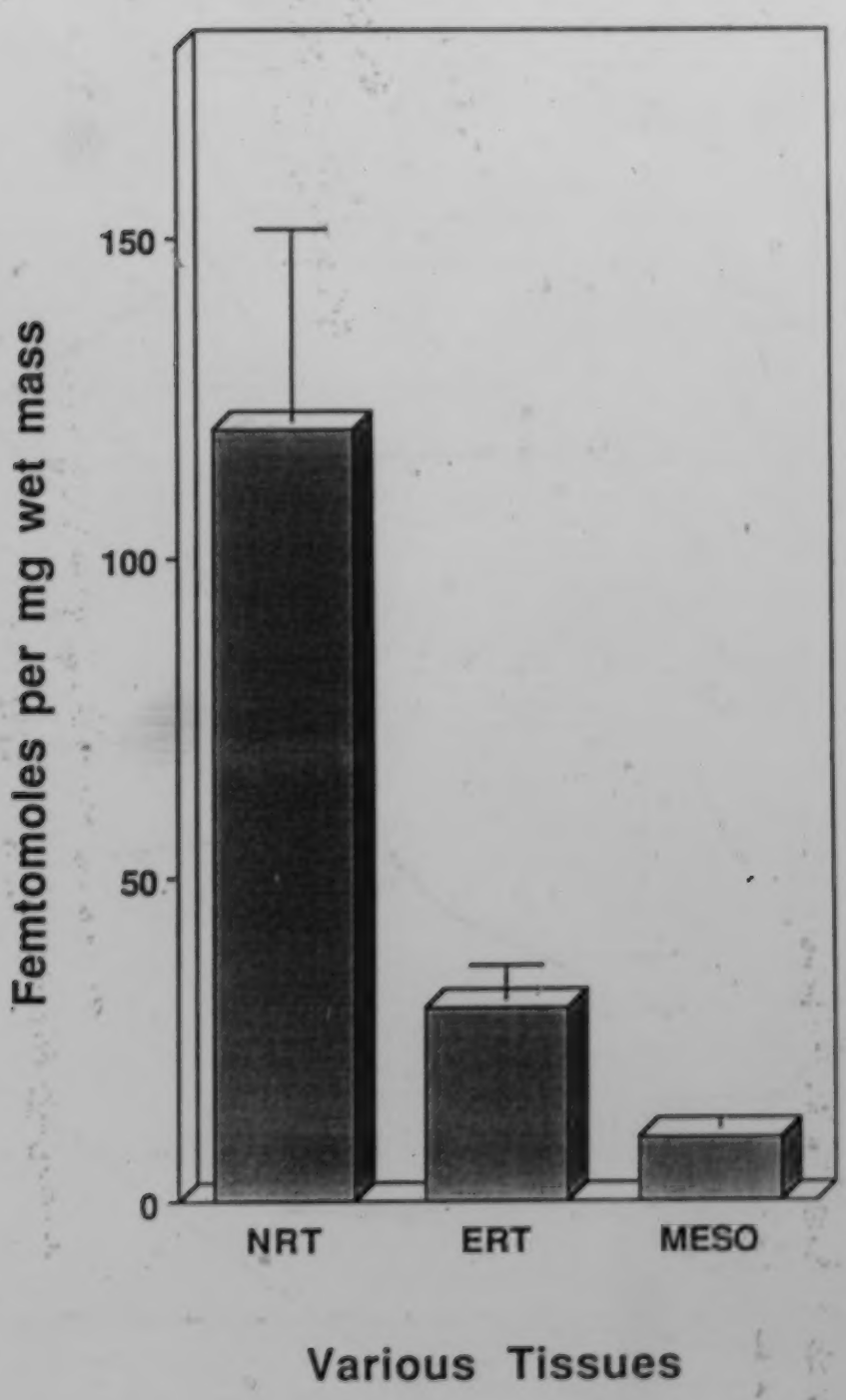
Any substance existing as a neurotransmitter can be expected to be found in relatively higher concentrations in nerve-rich tissues than in tissues having few or no neurons. In this respect, the higher concentrations of dopamine detected in tissue samples containing nerve-rings are probably significant.

Dopamine from endogenous or exogenous sources?

It was important to establish whether any of the detected dopamine came from an exogenous source such as food. Jellyfish were kept in a tank recirculating artificial sea

Figure II-17. Distribution of dopamine in *P. penicillatus*.

Concentrations of dopamine in alumina extracts of various tissues as determined using HPLC system II. Dopamine was found in the highest concentrations in NRT (nerve-rich tissue, N=10, 120 fmol/mg wet mass), at intermediate concentrations in ERT (endoderm-rich tissue, N=10, 30 fmol/mg wet mass), and the lowest concentrations in MESO (mesoglea, N=4, 10 fmol/mg wet mass). In this experiment, all animals were starved 2 days before use and tissue samples were collected under an anesthetizing saline (Mg^{2+}-containing sea water). *Error bars, S.E.M.*


Femtomoles per mg wet mass
150
100
50
0
NRT
ERT
MESO
Various Tissues

water and starved for various time intervals. The residence time of food in the hydrozoan gastrovascular system was assumed to be 24 to 48 h, since pseudofaeces were usually found in the tank 1 day after jellyfish were fed with brine shrimp. Starvation for 1 week apparently made jellyfish less active. The level of dopamine in the tissues examined increased ($p<0.05$) from 150 fmoles (N=10) to 180 fmoles (N=9) per mg wet mass after 3 days of starvation and then decreased ($p<0.005$) to 130 fmoles (N=10) which was close ($p=0.12$) to the control level (Fig. II-18). The level of dopamine would have decreased continuously with starvation if the dopamine were exogenous. It is, thus, highly probable that dopamine is an endogenous compound in this hydromedusa.

Effect of divalent cations on the content of dopamine in nerve-rich tissues.

Nerve-rich tissues were collected in different dissecting media; NASW, Ca^{2+}-free ASW, and ASW including Mg^{2+} or Ni^{2+} (obtained by mixing 0.33 M of $MgCl_2$ or $NiCl_2$ with ASW by 1:1 ratio). More dopamine was found in nerve-rich tissues collected in Ca^{2+}-free ASW or ASW including Mg^{2+} or Ni^{2+} than in tissues collected in NASW (Fig. II-19), indicating that the content of dopamine in the tissues was higher in the absence of Ca^{2+} than in the presence of Ca^{2+}. Calcium ions are known to be necessary for the release of neurotransmitters (Katz, 1966). Therefore, one possible explanation for this observation is that, during dissection, less dopamine would be released from dopaminergic cells in the absence of Ca^{2+} ions.

Figure II-18. Changes in the levels of dopamine in nerve-rich tissue during fasting.

The amount of dopamine in NRT was 150 fmol/mg wet mass (N=10) from animals which were sacrificed immediately after they were collected. Three days later the dopamine levels increased to 180 fmol/mg wet mass (N=9; $p<0.05$, *t-test*) and after six days returned to a level of 130 fmol/mg wet mass (N=10). During this experiment, jellyfish were not fed with brine shrimp and tissues were collected under an anesthetizing saline (Mg^{2+}-containing saline). *Error bars, S.E.M.*

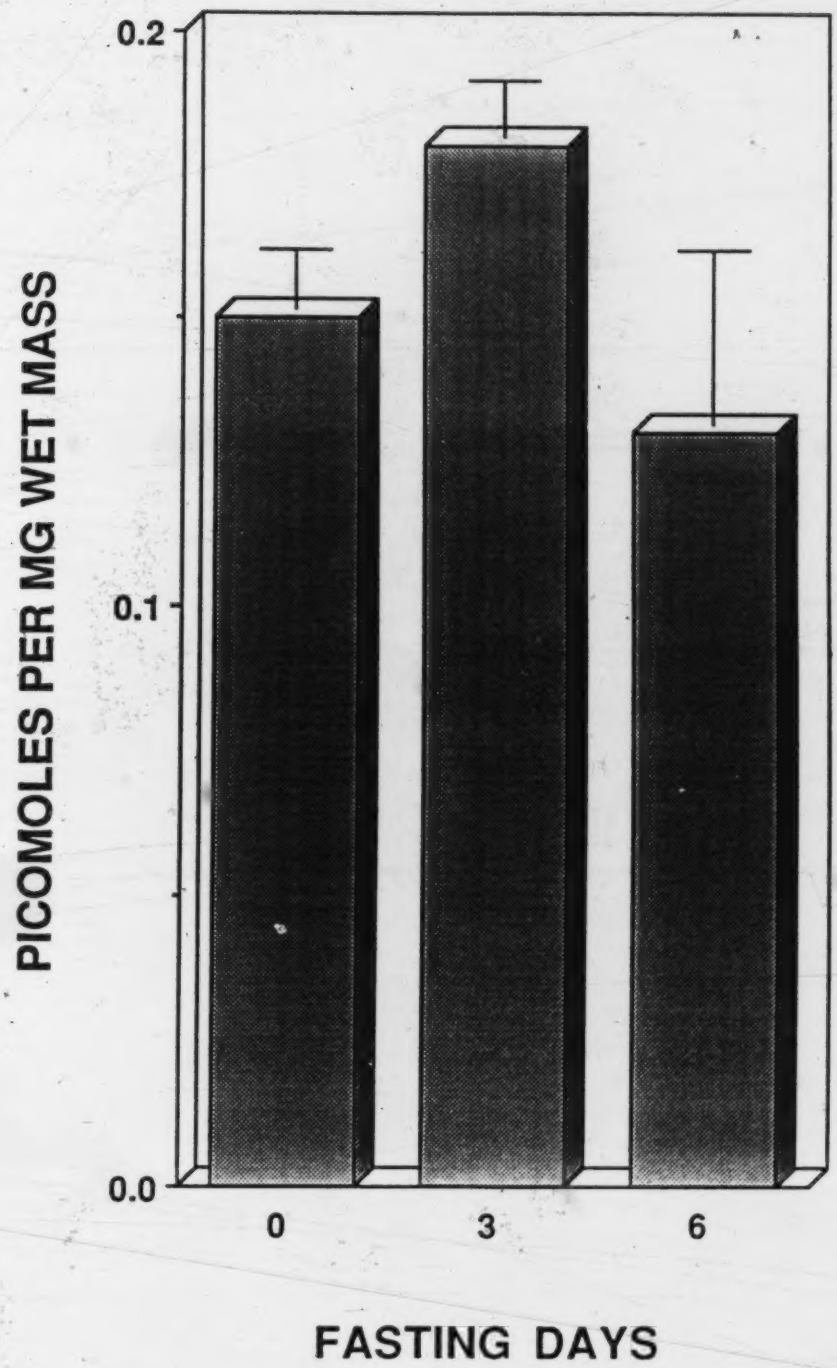
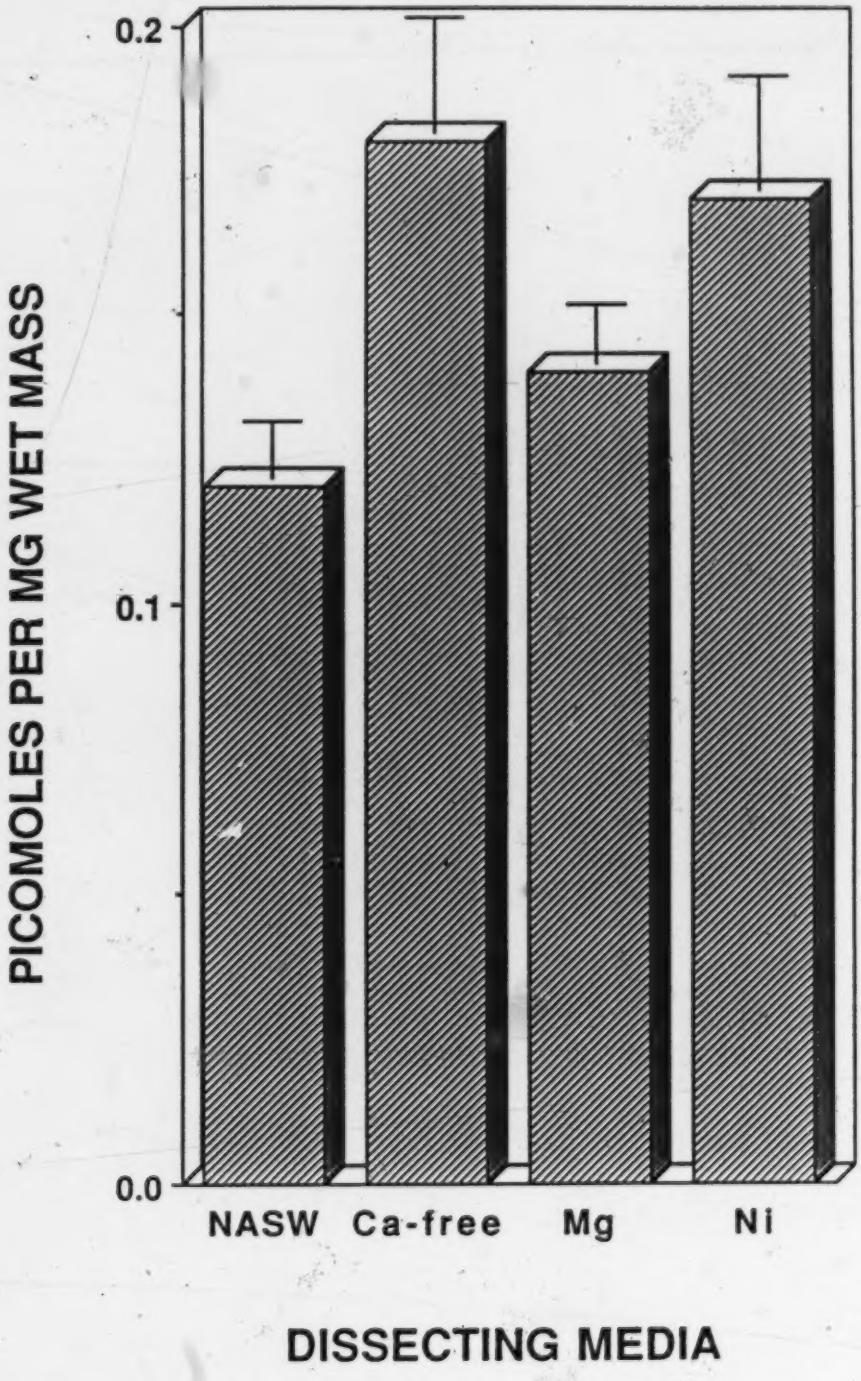

0.2
0.1
0.0
PICOMOLES PER MG WET MASS
0
3
6
FASTING DAYS

Figure II-19. Differences in the dopamine levels of nerve-rich tissue using different dissecting media.

When nerve-rich tissue (sample number N=14; 1 sample consisted of 3 animals) was dissected in normal artificial sea water (NASW), the concentration of dopamine was 120 fmol/mg wet mass. However, when dissected in Ca^{2+}-free saline or Ni^{2+}-containing saline, the dopamine levels were higher; 180 fmol/mg wet mass (N=10; $p<0.05$, *t-test*) and 170 fmol/mg wet mass (N=20; $p<0.05$) respectively. Tissue dissected under Mg^{2+}-containing saline showed a trend towards higher dopamine concentrations (140 fmol/mg wet mass, N=25) but the level was not significantly different from that in NASW. In this experiment, all animals were starved 2 days before use. *Error bars, S.E.M.*


0.2
0.1
0.0
PICOMOLES PER MG WET MASS
NASW
Ca-free
Mg
Ni
DISSECTING MEDIA

DISCUSSION

High performance liquid chromatography with electrochemical detection has become a popular technique for the identification and quantification of catecholamines and related compounds. This method combines a high sensitivity with precision and a wide linear dynamic range and can be made selective by altering the electrochemical conditions. There are, however, shortcomings with this method. In particular, it is difficult to identify and quantify any compounds which appear immediately after the solvent fronts of chromatograms. A second major problem occurs when unknown compounds have retention times that are identical to or very similar to compounds being used as standards. Under these circumstances unknown compounds may be misidentified. These problems may be circumvented by partially purifying the injected materials and by altering the components of the mobile phase. The first chromatographic system used, LC system-I, appeared to demonstrate the presence of norepinephrine, epinephrine, and dopamine in tissue samples of *P. penicillatus* (Fig. II-6B). Furthermore, the presence of these compounds seemed to be confirmed when addition of standard catecholamines to the samples incremented these peaks. A second system, LC system-II, was then developed to increase the degree of separation of the solvent front from the "norepinephrine" peak. The chromatograms from this system (Fig. II-7B) clearly showed that the "norepinephrine" peak of system-I was not norepinephrine, and that epinephrine, if present, was at very low levels. This system did nevertheless confirm the presence of dopamine in all tissue samples.

Further confirmation of the identities of the HPLC peaks was obtained using GCMS. In principle, the GCMS technique offers the greatest sensitivity presently available for the detection of trace organic compounds. However, when employed to detect endogenous compounds in a biological matrix, problems of increased chemical background and irreversible adsorption phenomena limit the application of this method to the nanogram range. In order to reduce the "noise" resulting from the chemical complexity of the tissues and to be consistent, I used alumina extracts as the source of catecholamines for the GCMS analysis. Alumina extracts were pooled to increase the amounts of catecholamines available for derivatization. The NICI mode of operation was chosen for

this study since Hunt and Crow (1978) have shown that this method can be 10 to 100 fold more sensitive for electron capturing compounds than when using the electron impact mode. Gas chromatography-mass spectrometry (GCMS) confirmed the presence of both dopamine and the internal standard (DHBA) in alumina extracts of nerve-rich tissues (Figs. II-13 & 16) but failed to detect any other catecholamines or DOPA. Peaks with retention times coincident with those of either DOPA or DOPAC appeared, though rarely, on LC chromatograms. HPLC combined with TLC showed the possible presence of DOPA in the nerve-rich tissue samples (Fig. II-10). It is possible that DOPA and DOPAC were not detected in GCMS since the recovery of these compounds from alumina extracts was too low to be detected.

It was not possible to identify the unknown peaks (X, Y) appearing on the LC chromatograms of alumina extracts, since the fragmentation patterns of the unknown compounds could not be interpreted from their mass spectra. The unknown compounds are probably catechol-derivatives for the following reasons. 1) These compounds must be electroactive within the potential range of the electrode material and solvent used. Catechols which have two hydroxy radicals in the benzene nucleus are known to be detected sensitively and selectively at the potentials (0.75 to 0.80V) used in this study (Mefford, 1985). Methoxylated metabolites of catecholamines require higher voltages to produce the response currents (Sternson et al., 1973). Tyrosine, having one phenolic hydroxyl group, requires a higher voltage than catecholamines (Sasa & Blank, 1977), indicating that the catecholamine's response to lower applied voltages are due to the dual oxidation of the hydroxyl groups on their benzene nucleus. The applied potential used in this study did not initiate the electrode response to octopamine and tyramine, and the unknowns are not expected to be phenolic compounds. 2) The alumina adsorption method is thought to be fairly specific for the catechol group, since the catechol moiety readily lends itself to adsorption on solid alumina (Anton & Sayre, 1962). Methoxylated metabolites of catecholamines and indoleamines, for example, were found to have extremly low recovery yields from alumina in our system. It will be of interest to identify the unknown compounds and examine their biological function. Direct coupling of HPLC with MS should be a useful technique for this identification.

High performance liquid chromatography has been used previously to identify catecholamines and related compounds in cnidarian tissues (Carlberg, 1983; Venturini

et al., 1984; Carlberg and Rosengren, 1985; De Waele et al., 1987). There appear to be considerable differences in the catecholamine contents of different species, with only meagre evidence for class-specific trends. Carlberg (1983) first reported the presence of DOPA, 5-OH-DOPA, and 5-S-cysteinyl-DOPA at extremely high concentrations in the sea anemones *Metridium* and *Tealia*; but surprisingly he did not detect any catecholamines. Nevertheless in a later study (Carlberg & Rosengren, 1985) of seventeen cnidarian species, fairly high levels of dopamine were reported in the scyphozoan, *Cyanea* (1.46 µg/g wet wt.) and the athecate hydrozoans *Hydra* (0.3 µg/g) and *Tubularia* (0.8 µg/g). These latter two species also contained fairly high levels, 0.03 and 0.3 µg/g wet weight respectively, of norepinephrine. In another hydrozoan polyp (*Hydra*), Venturini et al. (1984) found dopamine at 200 to 300 pmol/mg protein and norepinephrine at 100 to 300 pmol/mg protein. On the other hand, the hydromedusae *Aglantha* and *Eutonina* were reported by Carlberg and Rosengren (1985) to have very low levels of dopamine (0.003 and 0.004 µg/g wet wt., respectively). These concentrations are low when compared with the value (120 fmol/mg = 0.019 µg/g) obtained for dopamine in this study of the hydromedusa *P. penicillatus* (Fig. II-17). A greater concentration of dopamine (0.031 µg/g) was collected in the nerve-rich tissue samples when they were pooled under either Ca^{2+}-free or Ni^{2+}-added dissecting media (Fig. II-19). However, when one remembers that in this study nerve-rich tissues were used for extraction with alumina while in the previous studies whole animals were used, then these values become more comparable. The levels of dopamine found in the Hydrozoa were still lower than those found in other classes of cnidarians.

Although the purpose of the starvation experiments was to determine if the dopamine found in nerve-rich tissue was endogenous, it was surprising that the levels of dopamine increased after 3 days starvation. There is no obvious explanation for this finding. However, it is possible that the amounts of dopamine present in nerve-rich tissue varies in a cyclical manner (e.g. diurnal cycle) and the differences measured during starvation were merely due to samples being taken at different times during this cycle (Baker, pers. comm.).

It is interesting to compare the amounts of dopamine in the jellyfish with those in

mammalian tissues. The levels of dopamine in the jellyfish are apparently much lower than those of mammalian tissues except canine kidney, lung and mesenteric artery (Appendices 4 & 5). However, the level of dopamine in the jellyfish would be of the same order as that found in the mammalian heart. Considering the differences in the water content of each tissue, the amount of dopamine in the jellyfish would be 0.3 µg/g dry mass, and the amount of dopamine in the heart would be 0.76 µg/g dry mass. The dopamine level of mammalian brain tissue is around 0.9 µg/g wet mass (Appendix 4) and the percent of water content in the tissue is 75%. Thus, the dopamine level in the brain tissue is around 3.6 µg/g dry mass which is 12 times more than that in the jellyfish.

A number of histological studies, mostly using formaldehyde-induced fluorescence or glyoxylic acid-induced fluorescence, have demonstrated the presence of biogenic amines in neurons and other cells of cnidarians. Wood and Lentz (1964) reported norepinephrine and epinephrine in ganglion and sensory cells of *Hydra* which was later confirmed by Castàno and Rossi (1978). In the anthozoans biogenic amine-containing neurons have been reported by Dahl et al. (1963), Elofsson et al. (1977), and Van Marle et al. (1983) using histofluorescent techniques. Elofsson et al. (1977) and Van Marle et al. (1983) also reported the presence of biogenic amines in non-neuronal cells.

Many aspects of these studies are of particular interest with respect to our findings. First, it is apparent that several synthetic pathways for catecholamines have evolved in this phylum since the relative concentrations of the catecholamines and their precursors vary considerably between groups. For example, in the anemones, very high concentrations of DOPA are found, while in the Hydrozoa DOPA levels are quite low. It is possible that tyramine, and not DOPA, is the precursor for dopamine which is the dominant catecholamine in the hydrozoans. Second, many non-neuronal cells appear to contain catecholamines, though our study indicates that for *Polyorchis*, at least, nerve-rich tissues have a higher concentration of dopamine than other tissues. Although it may seem surprising that an acellular layer, the mesoglea, contains dopamine, the mesoglea must have the capacity to allow many types of organic molecules to diffuse readily through it. The mesoglea is the only pathway for nutrients to reach the exumbrella epithelium from the endodermal cells of the stomach and canal system. Perhaps the

mesoglea acts as a sink for the removal of dopamine after it is released. Van Marle et al. (1983) described the non-specific uptake of dopamine by the mesoglea in sea anemones and Anctil et al. (1984) have shown a similar phenomenon in *Renilla*. While Van Marle et al. (1983) reported that neither reserpine nor guanethidine affect the catecholamine stores in neurons, Anctil et al. (1984) have demonstrated that both epithelial and nerve cells in *Renilla* have mechanisms to take up norepinephrine and epinephrine. Finally, it can be expected that cnidarians synthesize a number of unknown catechols and catechol-derivatives. For example, Elofsson et al. (1977) described an unknown catechol derivative in tentacular neurons of anemones using microspectrofluorimetry. Carlberg and Rosengren (1985) suggested that this compound may be either DOPA or secoadenochrome.

Van Marle et al. (1983) reported that the formaldehyde-induced fluorescence seen in anemone neurons is due to a catecholamine resembling norepinephrine but that it is not genuine norepinephrine. Thus, there is some precedent for our finding an unidentified compound that has a retention time with HPLC that is similar to that for norepinephrine, but which is not norepinephrine, as determined by mass spectrometry.

In this study I have not localized dopamine to neurons although I have shown that nerve-rich tissues contain a higher concentration of this catecholamine than other tissues (Fig. II-17). However, the observation (Fig. II-19) that more dopamine was found in the nerve-rich tissues dissected and collected under either Ca^{2+}-free saline or the saline containing Ni^{2+} ions is significant, assuming that neurons were stimulated during dissection and released dopamine .

Some of the data presented in this chapter were published in 1989 by Chung JM, Spencer AN, and Gahm KH in "Dopamine in tissues of the hydrozoan jellyfish Polyorchis penicillatus as revealed by HPLC and GC/MS." J. Comp. Physiol. B. 159: 173-181.

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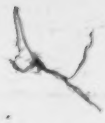

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CHAPTER III.
THE ELECTROPHYSIOLOGICAL EFFECT OF DOPAMINE ON SWIMMING MOTOR NEURONS OF THE JELLYFISH, *Polyorchis penicillatus.*

What can I wish to (sic) the youth of my country who devote themselves to Science? Firstly, gradualness. About this most important condition of fruitful scientific work I can never speak without emotion. Gradualness, gradualness, gradualness... Secondly, modesty...do not allow haughtiness to take you in possession...Thirdly, passion. Remember that science demands from a man all his life. If you had two lives that would not be enough for you. Be passionate in your work and your searching.

Pavlov.

[In 'The art of scientific investigation.' by Beveridge WIB (1957), pp.207-208, New York:Vintage Books.]

INTRODUCTION

In the previous chapter, dopamine (DA) was found as one of the main biogenic amines present in nervous tissues of *Polyorchis penicillatus*, using various methods of chemical analysis. It was also shown that dopamine was probably endogenously synthesized. However, such biochemical results do not give any information that can be used to determine the physiological role of dopamine. It is essential to verify that dopamine has a precise physiological action on target neurons before dopamine can be given serious consideration as a neurotransmitter or neuromodulator.

The biological role of monoamines in the Cnidaria has been examined in several studies (Table III-1). In several cases, exogenously applied catecholamines do not evoke any responses from target tissues despite catecholamines having been detected biochemically in these species (e.g. *Actinia equina, Calliactis parastica, Metridium senile;* See Table III-1). Other studies, however, present strong evidence that amines are acting as neuroactive substances. For example, Anctil et al. (1982) showed that monoamines control bioluminescence and contractile activity in the sea pansy, *Renilla köllikeri.* However, even these results are somewhat indirect and it is difficult to draw any conclusion concerning the true role of catecholamines in the Cnidaria. The lack of effect, as judged by changes in behaviour, when catecholamines were introduced into the bathing medium, does not necessarily imply that they have no neuroactive role in the animal. It is possible that the lack of effect was due to poor accessibility or rapid desensitization of receptors. Associated with the 'simple' morphology of the Cnidaria is the problem of gaining access to extracellular space around the target cells. It is apparent that most if not all neurons in hydromedusa are surrounded by ensheathing epithelial cells that are joined to each other by septate desmosomes (King & Spencer, 1979). These almost certainly act as effective diffusion barriers to any chemical agents applied to the external bath. It is only during the last decade that techniques have become available to permit 'direct' experimental analysis of the cellular effects of a transmitter substance. Until recently much of the data on cnidarian electrophysiology were obtained by extracellular recordings from intact or partially dissected animals. Interpretation of recordings obtained by extracellular suction or metal electrodes is particularly difficult

Table III-1. Physiological Studies of Biogenic Amines in Cnidarians.

Ross	*Calliactis parastica*	EN(?).
(1960)	*Metridium senile*	ACh, Histamine, 5HT, NE, DA, Tyramine; *all without effect.*
Lentz & Barrnett		
(1962)	*Hydra* sp.	ACh, NE, EN, 5HT, Histamine; *Nematocyst discharge.* Atropine, Tubocurarine, Hexamethonium; *Inhibit ACh effect on nematocyst discharge.*
(1963)	*Hydra* sp.	Antagonists (NE, EN, 5HT); *Inhibition of regeneration.*
Carlyle	*Actinia equina*	ACh, Carbachol, EN, DA, Tryptamine;
(1969)		*All without effect.*
Parmentier & Case	*Tubularia crocea*	Adrenergic mechanisms(?)
(1973)		(sympathomimetics)
Ball & Case	*Corymorpha palma*	Tyramine(+), Sympathomimetics;
(1973)		Ephedrine(+), Amphetamine(-).
Schwab	*Aurelia aurita*	Tyramine(-)
(1977)		
Erzen & Brzin	*Hydra viridis*	ACh; *no effect on its movement.*
(1978)		Atropine, Hexamethonium; *an inhibitory effect.*
Anctil et al.	*Renilla köllikeri*	NE, EN; *control luminescence & contractile activity.*
(1982)		
Hanai & Kitajima	*Hydra japonica*	DA & related amines (NE, tyramine)
(1984)		*depress the feeding response.*
Anctil	*Renilla köllikeri*	5HT & tryptamine
(1989)		*increase rhythmic activity.*
Anderson & Spencer	*Cyanea capillata*	ACh, EN, Oct., Nucleotides, Amino acids,
(1989)		& many peptides; *all without effect.*

-, *no effect.*

in the Cnidaria, since these electrodes record simultaneous activity from all excitable cell-types, such as nerve, muscle, and epithelial cells, in the vicinity of the electrodes. When intracellular recording techniques became available for some cnidarians such as a hydrozoan jellyfish *Polyorchis* (Anderson & Mackie, 1977), and a scyphozoan jellyfish *Cyanea* (Anderson & Schwab, 1982), it seemed likely that the direct cellular effects of putative transmitters and drugs could then be examined. Unfortunately, this did not prove to be so because of the accessibility problem mentioned above. It was not until the development of techniques (Anderson, 1985; Przysiezniak & Spencer, 1989) to isolate potential target cells that this became a realistic goal.

The aim of the present study was to elucidate the possible electrophysiological effects of dopamine on an identified neuronal type, the swimming motor neuron (SMN), from *Polyorchis penicillatus*. Furthermore I wished to determine the underlying ionic mechanisms of dopamine's action. Although there was no reason to suppose that SMNs were any more likely to be target neurons for dopamine than other neuronal types in the cultures, SMNs were used because their properties had been well characterised from *in vivo* studies and they were readily identified in culture. In the current-clamp mode, dopamine, ranging from 10^{-8} to 10^{-3} M, applied to cultured SMNs produced hyperpolarizations accompanied by a decrease of firing rate or complete inhibition of spiking produced by anodal break excitation. Dopamine in the voltage-clamp mode elicited outward currents at more positive levels than –55 mV, which was the reversal potential of the response. A series of ionic experiments suggest that the inhibitory effect of dopamine is caused by an increased permeability to potassium ions.

MATERIALS AND METHODS.

Medusae of *Polyorchis penicillatus* were collected from Bamfield Inlet, British Columbia, Canada by SCUBA divers, and held in running sea water (10-12 ^{o}C) for up to two weeks. They were fed once a week with brine shrimp.

A. Dissection.

Jellyfish were dissected by removing the apex and radially bissecting the bell. Single hemispheres were then pinned, subumbrellar-side uppermost, in an anaesthetic solution of 1:1 isotonic $MgCl_2$ and sea water to a Sylgard (Dow-Corning) base in a large Petri-dish using stainless steel pins and spines of the cactus *Opuntia*. The preparation was illuminated obliquely from below by a fiber-optic lamp. Figure III-1 shows the SMNs in a portion of the inner nerve-ring preparation of *P. penicillatus*. This preparation was used for either intracellular recording or collection of tissues for primary culture of SMNs.

B. Cell dissociation and culture.

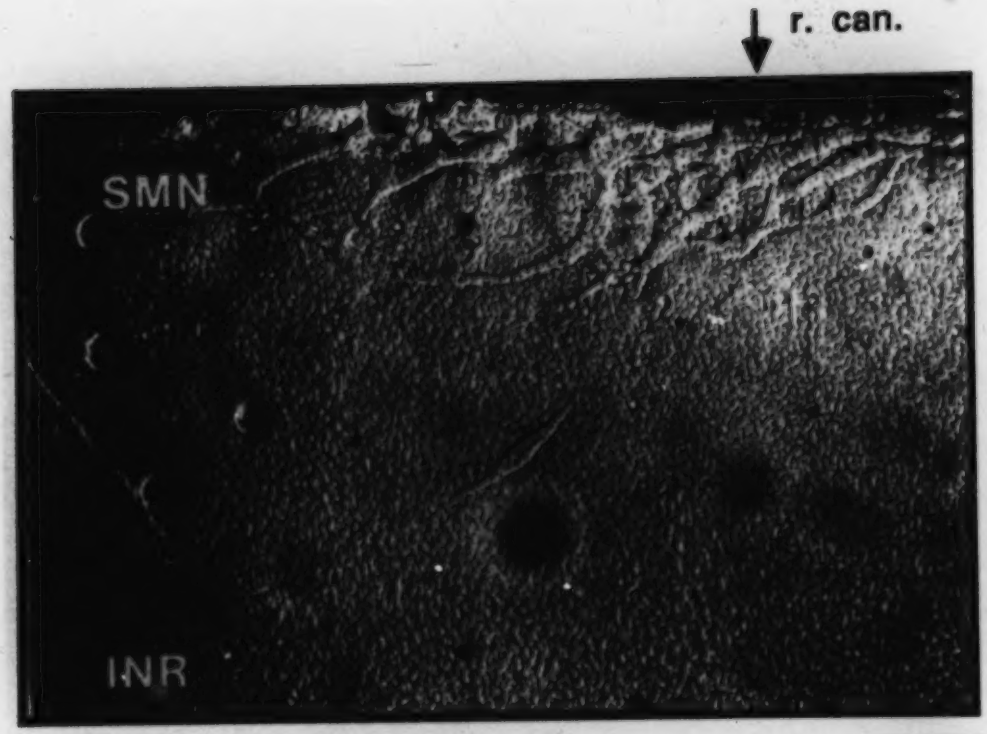
Principles of cell dissociation.

In spite of the diversity of techniques used to isolate cells from a variety of animals, there are some general principles involved (Trube, 1983).

1. Lowering the concentration of calcium ion: Many tissues are not completely dissociated by enzymes alone. Satisfying yields of isolated cells are only obtained if the calcium concentration is temporarily lowered during the process. This may be achieved by calcium chelators or by perfusion with calcium-free solutions. Electron microscopic studies show that calcium deficiency separates the outer portion of the basal lamina from the sarcolemma (Amsterdam & Jamieson, 1974).
2. Digestion by enzymes: Intercellular materials can often be dissolved by crude collagenase. Trypsin has also been used (Kostyuk et al., 1974). Proteolytic enzymes, however, are a potentially dangerous tool because they are known to affect membrane receptors (Lee et al,

Figure III-1. The network of swimming motor neurons.

Portion of the inner nerve-ring (INR) of *Polyorchis penicillatus* seen using Nomarski optics shows the network of swimming motor neurons (SMN). *r. can.* ring canal.


r. can.
SMN
INR
125µm

1977). In addition, trypsin was found to penetrate heart cells, causing severe damage to the cellular ultrastructure (Masson-Pevet et al., 1976). Hyaluronidase is sometimes added on the assumption that the mucopolysaccharides of the basal lamina are part of the intercellular cement (Glick et al., 1974). However, dissociation techniques employing collagenase alone seem equally effective (Isenberg & Klockner, 1982).

3. Mechanical agitation: In many tissues, tight and gap junctions are not (or are only partially) cleaved by enzymes and withdrawal of calcium. Some mechanical agitation is needed to dissociate the cells. Most commonly, the soft tissue is gently sucked up and down in a pipette.

Cell-cell and cell-substrate interaction *in vivo* play an important role in the control of cell migration, proliferation, and morphogenesis. Cell attachment to the surface is necessary for the application of many techniques *in vitro*. Several types of cells (e.g. fibroblasts) produce attachment factors and can be plated on glass or plastic surfaces. Other cell types (e.g. neurons) do not have the ability to produce attachment factors and require close contact with cells that do so or with specially treated surfaces that permit cell attachment. Many common methods of coating surfaces utilize components of the cell surface coat or the extracellular matrix (ECM) that have been implicated in adhesion (Hay,1981). These include collagen or gelatin, laminin, or fibronectin. Other methods use poly-amino acids, such as polylysine and polyornithine (Yavin & Yavin, 1974; McKeehan & Ham, 1976). Survival of isolated cells is markedly improved when the cells are placed on a feeder layer of fibroblast-like cells, or on a background layer of material prepared by lysing such cells and leaving the attached debris (Nishi & Berg, 1981), or when the surface is directly coated with ECM or basal lamina deposited by ECM-producing cells (Vlodavsky et al., 1980). Hence, dried mesoglea was used as a substratum in this study. Mesoglea is a complex mucopolysaccharide substance with collagen and elastin fibers running through it (Chapman, 1953a, b).

Procedures.

Neuronal cultures were prepared, as previously described (Przysiezniak & Spencer,

1989) with some modification (Fig. III-2) .

Strips of velum (approximately 30 mg wet weight) were removed from the inner nerve-ring, cut into small pieces, and placed in a borosilicate glass test-tube containing normal artificial sea water (NASW; 2 ml) for about 5 min. The NASW was replaced with double-distilled, deionized water for 30 s to 1 min, which removed many vacuolated epithelial cells due to hypo-osmotic shock. The tissue was rinsed again with NASW, and then exposed to divalent cation-free ASW (DFASW) for 10 min. Then followed a digestion in fresh collagenase solution (Sigma, Type I), 1200 to 1500 units/ml in NASW. After 6 to 8 hours of enzyme treatment at room temperature (around 18 to 20 ^{o}C), the enzyme solution was removed using a Pasteur pipette, and then 2 to 5 ml of NASW was added. The tissue was triturated 10 to 30 times with a fire-polished Pasteur pipette and the cell suspension divided among 10 culture dishes coated with homogenized, dried mesoglea. One hour after the cells were plated, the cultures were rinsed twice and covered with NASW, and then kept at 10-12 ^{o}C.

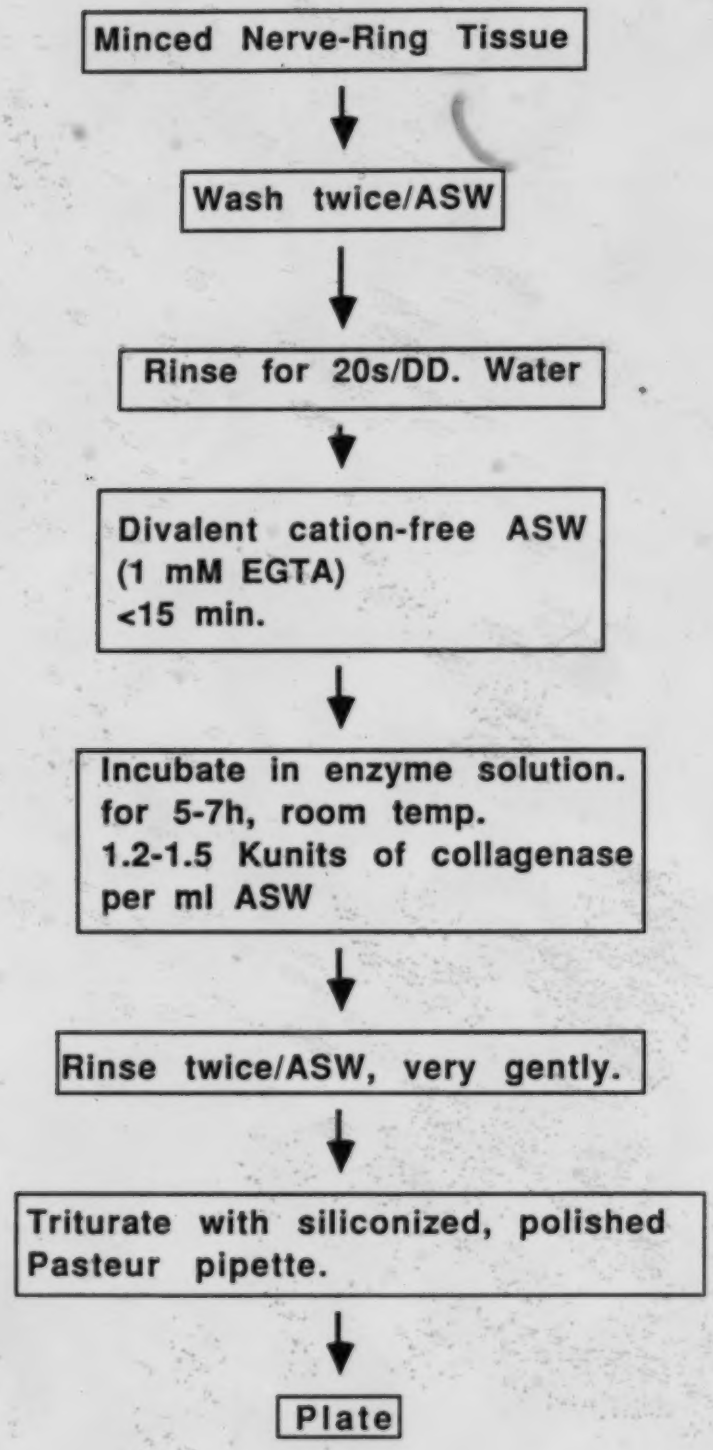
To prepare a mesogleal substratum, the bells from 3 to 5 animals were kept at room temperature (18 to 20 ^{o}C) for about 4 h in 500 ml of distilled water, changing the water every hour. The mesogleal lamella on the subumbrella side was then easily peeled off by pulling the ghost peduncle with forceps. The remaining exumbrella jelly was rinsed under flowing tap water, dissected into quarters, and kept at 4 ^{o}C in 500 ml of double distilled, deionized water for an hour. Cleaned mesoglea was placed on plastic Petri-dishes (Falcon 1008) or on glass slides and was dried flat at around 30 ^{o}C for 1 day in an incubator. Or homogenized mesoglea was used as a substratum. In this case, cleaned mesoglea from one animal was minced into several pieces, placed in about 25 ml of double distilled, deionized water, and blended at top speed for 20s (VirTis 23 blender) three times. 1.5 ml of the suspension was plated in each culture dish and left to settle at room temperature. After 1h, excess suspension was discarded, and the dishes were dried overnight at 30 ^{o}C in an incubator. Cultureware containing homogenized or whole mesoglea substratum was kept at 4 ^{o}C in a refrigerator until used.

C. Whole cell recordings (WCR).

Recordings from cultured neurons were made with a List L/M-EPC 7 amplifier (Medical Systems Corp., Greenvale, NY) and were obtained in either voltage or current-

Figure III-2. A flowchart of the procedure used for dissociating the nerve-rich bell margin of *Polyorchis penicillatus*.

ASW, artificial sea water; DD water, Double-distilled Deionized water.


Minced Nerve-Ring Tissue
Wash twice/ASW
Rinse for 20s/DD. Water
Divalent cation-free ASW
(1 mM EGTA)
<15 min.
Incubate in enzyme solution.
for 5-7h, room temp.
1.2-1.5 Kunits of collagenase
per ml ASW
Rinse twice/ASW, very gently.
Triturate with siliconized, polished
Pasteur pipette.
Plate

clamped mode using the WCR configuration (Fenwick et al. 1982).

Patch pipettes were made on a Narishige electrode puller PP-83 (Narishige, Tokyo) using non-heparinized hematocrit capillary tubes (Fisher; length 75 mm, ID 1.1-1.2 mm, thickness 0.2 mm). Their resistances were 1.0 to 3.0 MΩ when patch solution (PS) and NASW listed in Table III-2 were used as the patch pipette solution and bath solution respectively. Careful attention was paid to obtain "Giga-ohm" seals. When the tip of the pipette was depressed against the SMN surface, the seal resistance was 20 to 50 MΩ (the resistance was measured by applying a 1 mV voltage pulse in the pipette and monitoring current flow). When a mild negative pressure was applied, the seal resistance increased rapidly from 20-50 MΩ to a higher value; 500 MΩ in the best cases. The pipette potential was then changed to a negative voltage (approximately 60 mV below the bath potential) and repetitive hyperpolarizing voltage steps of 5 or 10 mV were given to the cell, resulting in a rapid increase of the seal resistance. The patch membrane was very often ruptured at this stage and it was not possible to measure the seal resistance before gaining access. Without immediate rupture, the seal resistance often increased to 1 GΩ or more at –60 mV of membrane holding potential. Interestingly, a leak resistance measured by a 10 mV hyperpolarizing pulse after break-through was also drastically increased to 1 to 2 GΩ at a holding potential of –60 mV. An increment of the apparent leak resistance mirrors an increment of the seal resistance (see Appendix 6).

The following criteria for whole-cell recordings were used:
(1) leak resistance (see Appendix 6) had to be larger than 0.8 GΩ (usually 1 GΩ; Max 2.5 GΩ); (2) the resting potential had to exceed –25 mV (usually –30 mV) initially and the change of the resting potential had to be less than 5 mV during the experiment; (3) the amplitude of the rebound action potentials had to reach at least 80 mV. The recordings were accepted for analysis when all three criteria were satisfied.

The series resistance of the pipette tip was measured by two methods: (i) the amplitude of a voltage step was divided by the peak current recorded at the onset of the capacitive transient and (ii) the time constant of the transient was divided by the membrane capacitance. Membrane capacitance was obtained from time constants of either depolarizing or hyperpolarizing responses to current pulses in the current clamp

Table III-2. Ionic Composition of Salines.

	NASW	DFASW	High K^+	V.High K^+	low-Cl^-	PS
NaCl	378.0	378.0	378.0	328.4	378.0*	-
$CaCl_2$	9.5	-	9.5	9.5	9.5	1.0
Na_2SO_4	5.7	5.7	5.7	5.7	5.7	-
KCl	13.4	13.4	55.4	105.0	13.4*	105.0
$MgCl_2$	29.0	-	29.0	29.0	29.0	2.0
ChoCl	42.0	99.0	-	-	-	-
HEPES	10.0	10.0	10.0	10.0	10.0	10.0
NaOH	5.0	6.0	5.0	5.0	4.0	-
EGTA	-	1.0	-	-	-	11.0
glucose	-	-	-	-	93.5	700.0
KOH	-	-	-	-	-	35.0
mosmol	1019.4	1019.4	1019.4	1019.4	1026.9	1010.0

Gentamycin sulfate was added to all saline solutions [$5x10^{-3}$ (w/v) %] except patch solution (PS).

*. Both salts were replaced with Na and K-gluconate respectively.

EGTA, ethyleneglycolbis(oxyethylenenitrile)tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; ChoCl, Choline chloride; NASW, normal artificial sea water (K^+ 13.4, Cl^- 510.4 mM); DFASW, divalent cation-free artificial sea water; High K^+, high potassium ion (55.4 mM) saline; V.High K^+, very high potassium ion (105 mM) saline; low-Cl^-, low chloride ion (77 mM) saline.

mode. Access resistance values from 5 to 10 MΩ were routinely obtained, and 20 to 50% of series-resistance could be cancelled using the slow capacity compensation of the patch-clamp amplifier. With access resistances of 3 to 5 MΩ, currents of 1 nA (the largest that applied dopamine evoked in this study) would have caused no more than a 5 mV voltage drop across this resistance. I therefore chose to neglect series resistance errors in the analyses of the results.

Junctional potentials were nulled by an offset sufficient to make the output current zero when the pipette was in the bath. The maximum potential drift observed when junction potential was checked after recording from cells was 2 mV. The junctional potentials did not vary by more than 1 mV when the bath was perfused with the various test solutions listed in Table III-2. I did not correct for this error.

Intracellular voltages and currents were recorded continuously on a Gould Brush 2400 pen recorder and for later analysis stored on VCR tapes using a VCR (MTS, model MCR-220R) and an A/D VCR recorder adapter (PCM-2, Medical Systems Corp.). Recorded signals were displayed on a Tektronix 5223 digitizing oscilloscope. Some of the voltage clamp data were acquired and analyzed with an IBM-PC equipped with a Labmaster TL-1 interface (Axon Instruments) controlled by pClamp software (version 5.03).

D. Preparation of solutions

The compositions of several bathing solutions and a patch solution are listed in Table III-2. Dopamine solutions of 1 mM to 10 nM in NASW were prepared daily by dilution of stock solutions prior to use. The stock solutions of dopamine (0.1 M) were prepared in deionized, double-distilled water, and were frozen at –20 °C after being divided into aliquots of 500 ml. The pH of the stock solution was 6.8, and the pH of the working dopamine solutions was 7.5. The working dopamine solution in NASW oxidized relatively easily, becoming a brownish pink colour. Such autoxidation of dopamine could have been prevented by adding ascorbic acid. Ascorbic acid, however, was not used as an antioxidant in these experiments to avoid any possible interfering effect of pH (Anderson and McKay , 1987 ; Spencer, 1988). Instead, the working dopamine solutions were enclosed in aluminum foil, kept in a dark-box at 4 °C, and prepared freshly every 4 hours during

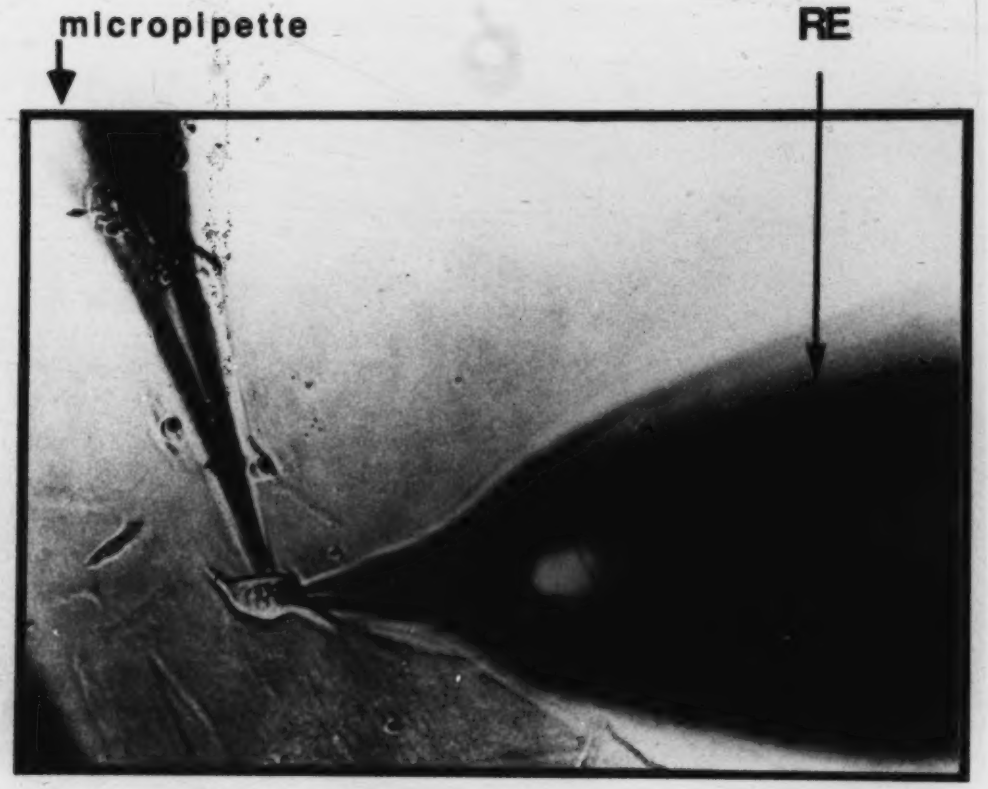
the experiment. Dopamine solutions were introduced into pipettes using a hypodermic 'stainless steel' needle. Dopamine was purchased from SIGMA Chemical Co. (St. Louis, MO); other chemicals were of reagent grade and were obtained from local suppliers.

E. Dopamine application

Dopamine dissolved in NASW was delivered to isolated cells via capillary pipettes. Pipettes were pulled in two stages from aluminosilicate glass (AM Systems, OD 1.5 mm, ID 0.58 mm) in the same manner as for the WCR. Pipettes obtained when the heat was reduced to about the breaking point were used (their bubble numbers were from 6.2 to 6.5). The pipette was positioned 10 to 50 µm from the target soma, with the tip of the pipette at an angle of about 45 degrees to the perfusion-path (Fig. III-3). A fluid stream from the pipette was obtained by applying 10 to 20 psi (69-138KPa) pressure to the pipette using a Picospritzer (General Valve Corp., Model-2). The concentration of dopamine in the vicinity of the cell body depended on several factors including diffusion. Thus the dopamine concentration in the pipette represents only an upper limit for the concentration that could have reached the surface of the cell. The amount of dopamine ejected was calculated by determining the ejected volume at the end of each experiment (Sakai et al. 1979).

Figure III-3. Arrangement for recording agonist-induced responses from a swimming motor neuron.

Agonists were applied to the soma with a micropipette (methanol bubble number 6.2) attached to a Picospritzer. *RE* recording electrode.


micropipette
RE
100μm

RESULTS

A. Primary culture and identification of swimming motor neurons.

It was not until the use of mesoglea as an ECM for cell culture (Schmid & Bally, 1988) that jellyfish neurons were sucessfully cultured. Neurons from *P. penicillatus* did not attach to the plain Petri-dishes as well as to those coated with a known ECM such as gelatin, collagen or poly-L-lysine. However, the best attachment was obtained using culture dishes coated with dried mesoglea pieces or dried mesogleal homogenates (Przysiezniak & Spencer, 1989).

Swimming motor neurons could be distinguished morphologically and electrophysiologically from other subsets of neurons *in vitro*. The morphological criteria used to identify SMNs in primary culture were; a clear cytoplasm, short and wide processes, and membranous inclusions around the nucleus (Fig. III-4A). Neurons identified as SMNs maintained a distinctively shaped action potential in culture (Fig. III-4B). The amplitudes of action potentials in culture (80-100 mV) using WCR were similar to those recorded *in vivo* using intracellular recording technique (Satterlie & Spencer, 1983). Spike duration at half amplitude ranged from 20 to 50 ms. Afterhyperpolarizations with amplitudes of –10 to –15 mV followed each spike. Neurons exhibiting spikes with these characteristics were generally larger (somata > 30 μm) than other neurons, however, some were as small as 10 to 20 μm. Only cells showing these distinctive characteristics were used in this study.

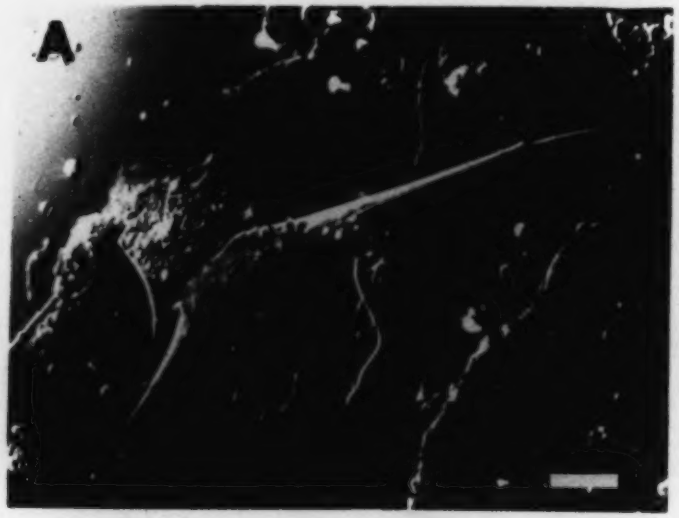
B. Whole cell recording.

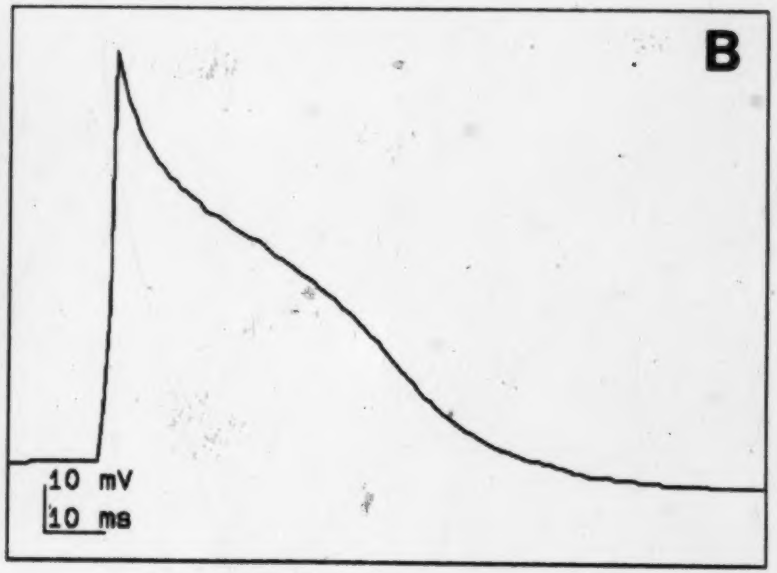
Intracellular voltage recordings were obtained by switching from the voltage clamp (VC) mode to the current clamp (CC) mode in the WCR configuration. Measurement of the resting membrane potential with standard solutions gave a mean of –30.0 ± 1.2 mV (n=30, mean ± S.E.M. ; range from –48 to –20 mV). These values are different from those obtained from either *in vivo* or *in vitro* preparations using intracellular microelectrodes. Anderson & Mackie (1977) measured an average *in vivo* resting potential for SMNs of –60 ± 5 mV, Spencer (1981) measured a mean of –57 mV, and I (1988, unpublished) measured a mean of –48 ± 3 mV (n=10) using intracelluar

Figure III-4. Identification of swimming motor neurons (SMNs) in cell culture.

A. A cell, marked with an asterisk, viewed by Nomarski differential-interference-contrast microscopy shows the clear cytoplasm and perinuclear membranous structures typical of SMNs. Scale bar, 50 μm.

B. An action potential elicited by current stimulation (2.5ms/999pA) from the same cell as in **A** while holding the resting membrane potential at -35 mV. Note the characteristic plateau of the SMN. Bath contained in mM; NaCl 378, $CaCl_2$ 9.5, Na_2SO_4 5.7, KCl 13.4, $MgCl_2$ 29, Choline chloride 42, HEPES 10, NaOH 5. Patch pipette contained in mM; $CaCl_2$ 1, KCl 105, $MgCl_2$ 2, HEPES 10, EGTA 11, glucose 700, KOH 35. Both solutions had a pH 7.5.


A


B
10 mV
10 ms

microelectrodes. Such values may not correspond to the true cell resting membrane potential for the following reason. All excitatory and inhibitory inputs to the SMN network might not be completely blocked in the *in vivo* preparation, resulting in fluctuations of the resting potential. Measurement of resting potentials from an isolated neuron should circumvent such a problem. Przysiezniak & Spencer (1989) measured a mean resting potential of -53 ± 20 mV from isolated SMNs using intracellular microelectrodes. It is apparent that cell membrane damage may be more frequently associated with intracellular microelectrode recording from *in vitro* preparations than from *in vivo* preparations. For example, the amplitudes of spontaneous spikes in isolated SMNs (Przysiezniak & Spencer, 1989) were reported to be lower than those from neurons *in vivo*, with mean values of 50 mV *vs* 90 mV respectively. Also, the measured resting potentials from the *in vitro* preparation have far greater variance than those from cells *in vivo*.

Whole-cell recording may circumvent these errors, however this does not necessarily mean that the resting potentials measured by WCR represent the actual resting membrane potential. The calculated equilibrium potentials for potassium and chloride are –59.1 and –38.4 mV at 20 ^{o}C respectively on the basis of the concentrations of each ion in the bath and patch solutions. The measured resting potential should have been close to the calculated equilibrium potential for potassium assuming that solution exchange occurred completely between the electrode solution and cytoplasm, and that the resting potential was determined by the potassium concentration gradient across the membrane. This suggests thai resting potentials in SMNs may not be based on the potassium ion gradient alone. Also, since the normal intracellular milieu may be significantly altered by cytoplasmic dialysis, measurements of resting membrane potential using patch electrodes would be in error if other cellular components as well as ions participated in determining the resting potential of a cell.

C. Characteristics of the dopamine response in swimming motor neurons.

Current clamp mode. Switching from VC to CC evoked 'anodal break' spikes when neurons were held at –60 ± 0.2 mV in the VC mode. The presence of long-duration spikes (more than 20 ms) confirmed that the neurons were SMNs.

Pulses of dopamine applied to SMNs during the anodal break excitation produced hyperpolarizations which were associated with a decrease of the firing rate or complete inhibition (n=15; Figs. III-5A and B). Control applications of normal artificial sea water (NASW), which was used as a dopamine vehicle, did not normally produce any responses (n=5). In some cases when the leak resistance was less than 500 MΩ, NASW caused depolarization (less than 3 mV) with a slight increase in firing frequency.

The shapes of action potentials before and after dopamine-induced hyperpolarization are shown in Fig. III-5C. Spikes just following the hyperpolarization usually had slightly greater amplitudes and shorter durations than spikes before and tens of seconds after the dopamine-induced hyperpolarization. It is interesting that increases in spike amplitude were greater in recordings from cells in which the amplitudes of the action potentials were developed due to a 'low' resting potential which may have resulted from cell damage by the electrode, are transformed by dopamine into 'healthy' looking action potentials of large amplitude and short duration. The rising phases of action potentials were not altered by the application of dopamine.

Dopamine also elicited a decrease in the input resistance of neurons. A series of current pulses of 2s duration and 10 pA incrementing-steps from –30 to 0 pA produced anodal break spikes following passive hyperpolarizing responses and spontaneous spikes from a SMN in NASW (Fig. III-6A). However, current pulses from –40 to 10 pA failed to generate a spike and the hyperpolarizing steps were of decreased amplitude in the presence of dopamine (Fig. III-6B). After washing, the cell recovered its excitability (Fig. III-6C). From the relationship of the current stimulations to the voltage traces, the input resistance of the cell was calculated to be 1.42 GΩ (Fig. III-6D). The input resistance changed from 1.42 to 0.68 GΩ in the presence of 10 μM of dopamine and returned to its control value after washing.

The decrease of input resistance could be due to a direct action of dopamine or indirectly by altering the membrane potential. To examine this, the membrane potential was manually manipulated by injecting direct current into a cell in order to simulate hyperpolarization by dopamine (Fig. III-7). The hyperpolarization due to DC current did not decrease input resistance while dopamine at 10 nM elicited membrane hyperpolarization and a decreased input resistance. Thus, the decrease of input resistance in the presence of dopamine was due to dopamine itself, and not to the change

Figure III-5. The effects of dopamine on the rebound action potential trains from cultured swimming motor neurons.

A. Either saline or 10 μM of dopamine in saline were applied (shown by the bar) through a micropipette (bubble number 6.2) using a 'Picospritzer' (pressure 138 KPa) to a SMN. All recordings in A were made consecutively. 1). Control experiment in which saline was applied for 2s. 2). Dopamine applied for 2s resulted in membrane hyperpolarization and a dramatic reduction in the firing frequency. 3). A second application of dopamine 3.2 mins later for 2s produced only a slight reduction in firing frequency as a result of desensitization. Saline-perfusion started at the arrow. 4). Dopamine applied for 4s evoked distinct membrane hyperpolarization and reduction or cessation of firing.
Note that the first spike to appear after dopamine application had a higher amplitude than the spike prior to dopamine application.

B. 1). Control experiment in which saline was applied for 4s. 2). Application of dopamine at 10 μM for 4s provoked a long hyperpolarization (peak amplitude, 15 mV; duration, 26s) from this cell. This is a different cell from that in **A**.

C. Digitized single sweeps obtained from the rebound spike trains in figure 1A-4. **a** shows a spike just before dopamine application, **b** shows a spike appearing immediately after dopamine application, **c** shows a spike 2.8 mins after dopamine application.

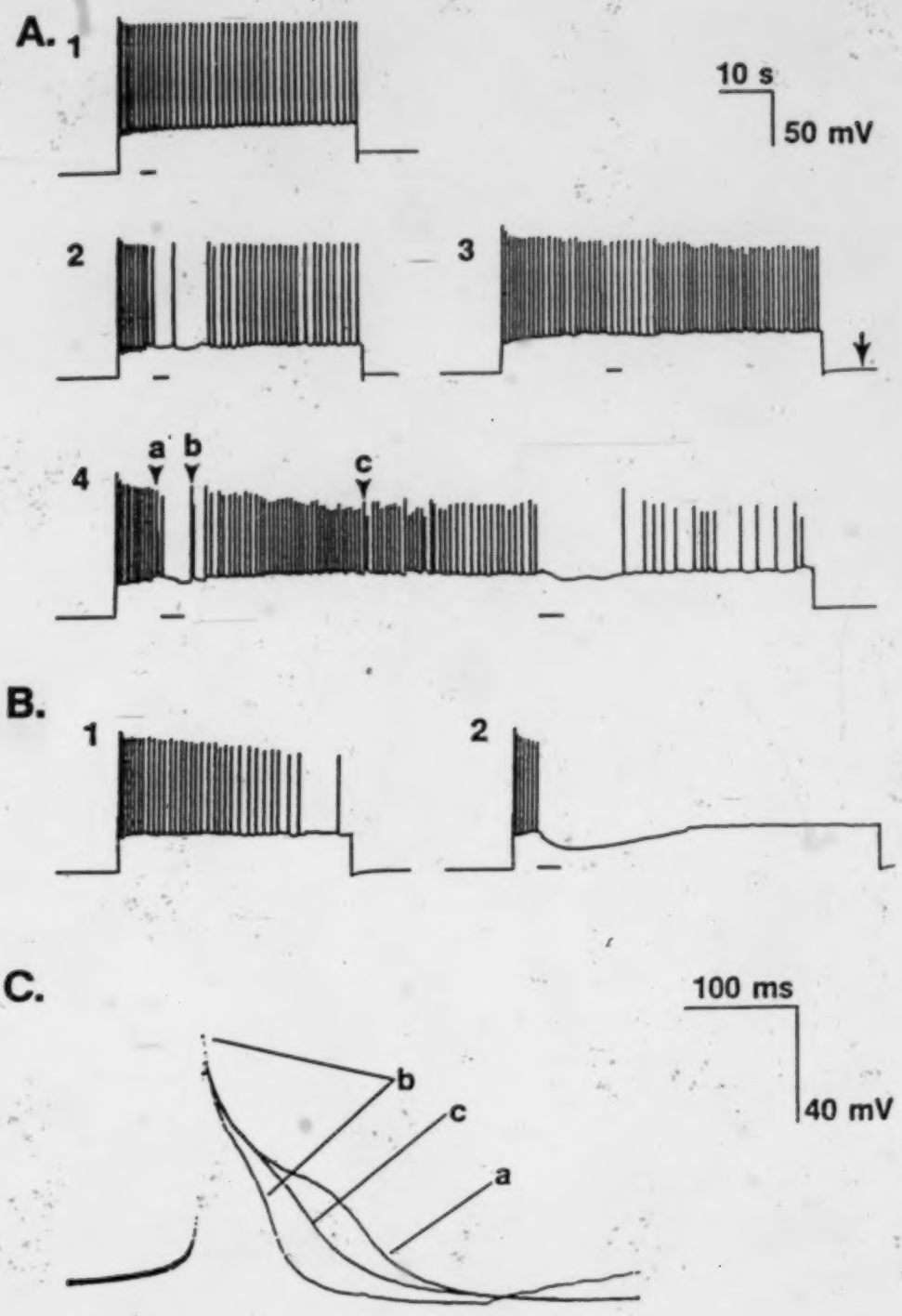

A.
1
10 s
50 mV
2
3
4
a
b
c
B.
1
2
C.
100 ms
40 mV
b
c
a

Figure III-6. The effects of dopamine on the input resistance of swimming motor neurons.

A. Control voltage traces produced by a series of current pulses of 2s duration starting at –30 pA, increasing in 10 pA steps. The responses to negative current stimulations of 10 pA or more produce anodal break action potentials.

B. In the presence of dopamine (10 μM), the same current pulses produced smaller voltage deflections and did not generate any anodal break discharges. Dopamine (DA) also hyperpolarized the basal membrane potential.

C. Voltage-traces to a current pulse of –10 pA under control conditions, with dopamine, and after washing. Digitized sweeps for the CTL (control) and dopamine were reconstructed using the hyperpolarizing responses to a current step of –10 pA appeared in **A** & **B**. In the presence of dopamine, the base membrane potential was drastically hyperpolarized from –28 mV to –42 mV and the hyperpolarizing response to the current pulse was decreased, implying that the input resistance decreased.

D. Voltage values at 500ms after the start of current pulses were selected to measure input resistances. The input resistance changed from 1.42 to 0.68 GΩ by dopamine and returned to its control value after washing (1.42 GΩ).

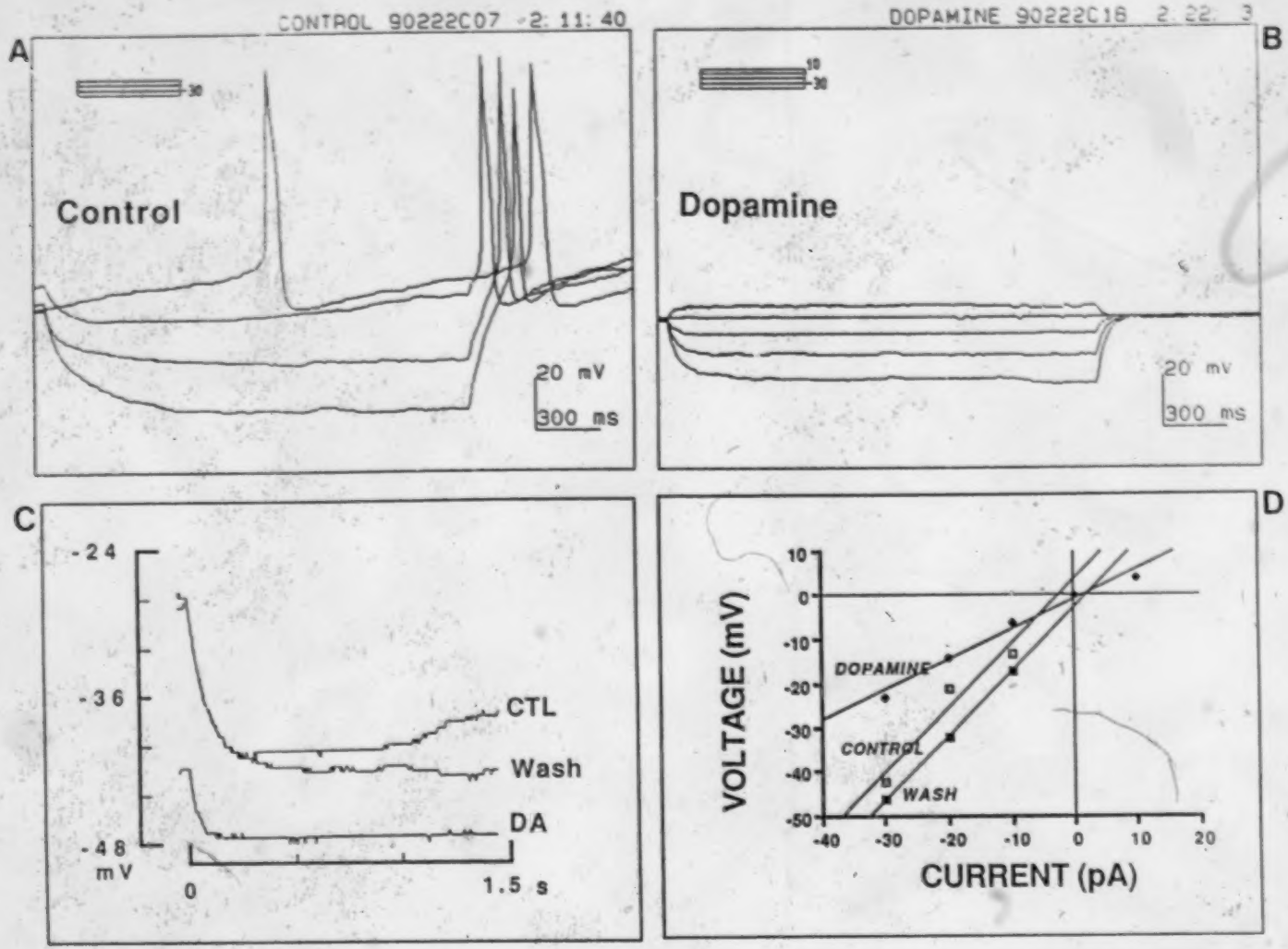
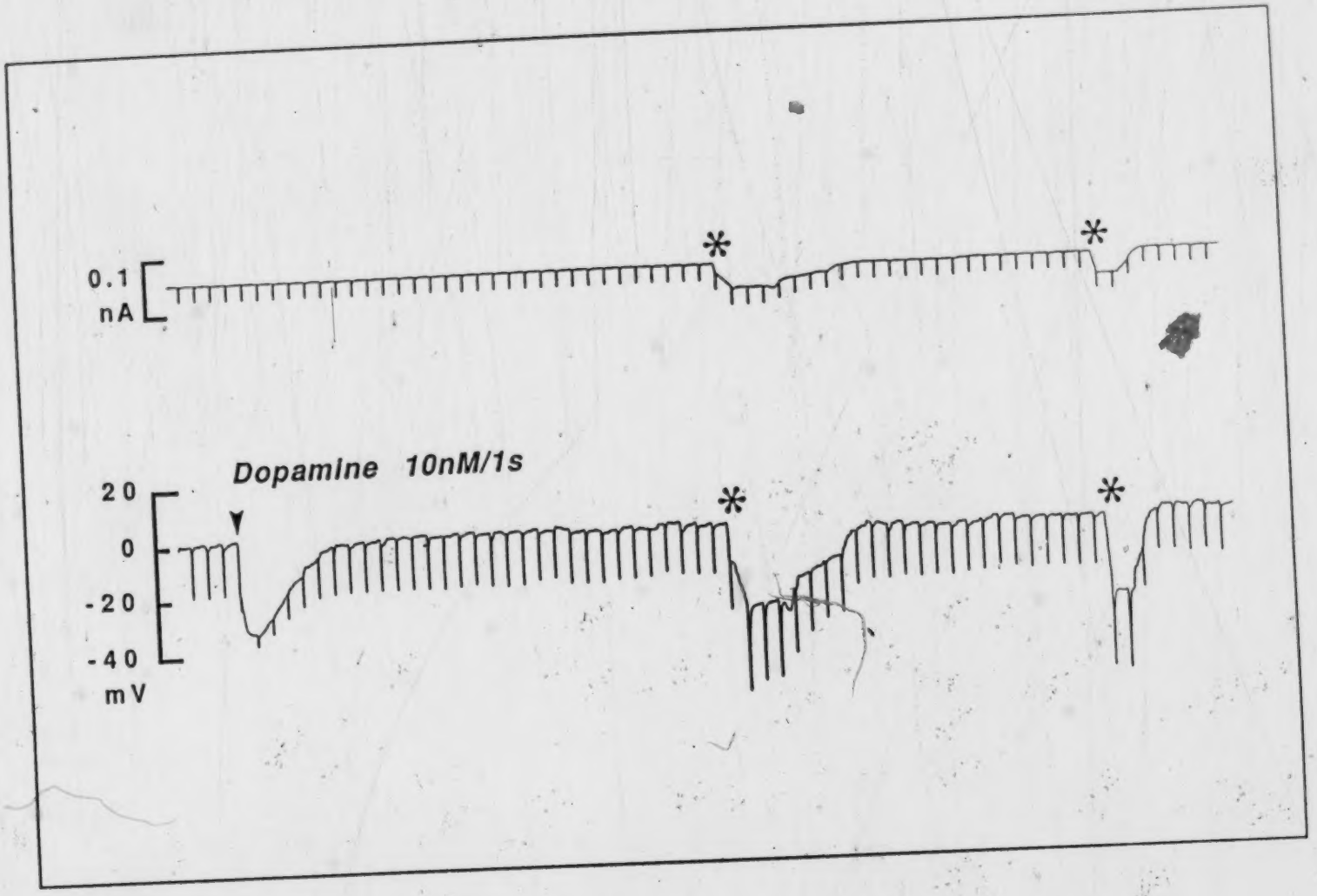

A
CONTROL 90222C07 2: 11: 40
Control
20 mV
300 ms
B
DOPAMINE 90222C18 2 22 3
Dopamine
20 mv
300 ms
C
-24
-36
-48
mV
CTL
Wash
DA
0
1.5 s
D
VOLTAGE (mV)
CURRENT (pA)
DOPAMINE
CONTROL
WASH
10
0
-10
-20
-30
-40
-50
-40
-30
-20
-10
0
10
20

Figure III-7. The decrease of input resistance when dopamine is applied is a direct effect and is not due to membrane hyperpolarization.

Ten nM dopamine generated a 34 mV hyperpolarization and a marked decrease of input resistance in a cell which was held at 0 mV (lower trace). Direct current was intracellularly injected (starred) to mimic the membrane hyperpolarization (upper trace). The hyperpolarization due to injected DC current did not "decrease" the input resistance. This indicates that the change of input resistance in the presence of dopamine was not a consequence of the change of membrane potential but was due to dopamine itself. Current pulses were applied at 0.35 Hz, 30 pA, and 200 ms duration.


0.1
nA
Dopamine 10nM/1s
20
0
-20
-40
mV

in membrane potential.

In fact, the input resistance of the neuron increased as the cell became more hyperpolarized (Fig. III-8). Hyperpolarizing current pulses of 30 pA at a frequency of 0.35 Hz were injected through the electrode into cells in which the membrane potential was held from –40 mV to 0 mV. When a cell was held at –30, –20 and 0 mV, 10 nM of dopamine drastically decreased apparent input resistance by 80, 85, and 93% respectively, at the peak of the dopamine-induced hyperpolarizations (Figs. III-8B, C, D).

Voltage-clamp mode. Applications of dopamine at concentrations ranging from 10^{-8} to 10^{-3} M evoked monophasic outward currents when the membrane potential was held at more depolarized levels than –50 mV (the inset of Fig. III-9). All SMNs (n=56) responded to applied dopamine by producing outward currents when the recording criteria were met and the pressure-ejection pipettes were positioned within 100 μm of the soma. Neurons which gave short-duration spikes and were provisionally identified as 'B' neurons (Spencer & Arkett, 1984) did not respond to dopamine (n=6). Control applications of saline either did not produce a response or gave responses which did not resemble those due to dopamine (n=8). Three such control applications gave small inward currents (<20 pA) whose amplitudes were not dependent on membrane holding potential.

D. Voltage dependence of dopamine-induced outward currents.

The response evoked by dopamine was strongly dependent on membrane potential, showing a current-voltage plot with outward rectification as the membrane was depolarized (Fig. III-9). The amplitude of the response to dopamine varied non-linearly with the clamped membrane potential in all cells studied. The reversal potential of the response was –55.0 mV (SEM= ±1.1mV; n=11).

The amplitudes of the responses seen in different cells varied between 150 to 500 pA at 0 mV. This variation was neither related to cell size nor to the age of the culture. Conductances were calculated from samples whose peak amplitudes were between 150 and 200 pA at 0 mV. Slope conductance measurements, obtained from tangents to the

Figure III-8. Voltage-dependency of the change in the input resistance of a swimming motor neuron induced by dopamine.

Thirty pA hyperpolarizing current pulses of 200 ms were applied at a frequency of 0.35 Hz to a cell in which the holding potential was varied from –30 to 0 mV. Ten μM of dopamine was applied for 1s using a 'Picospritzer' (pressure 138 KPa). **A** shows the stimulating current traces and **B**, **C**, and **D** show the changes of the input resistance in the presence of dopamine at holding potentials of 0, –20, –30 mV respectively. Note that as the holding membrane potential becomes more depolarized, the hyperpolarizations increase and the input resistance decreases.

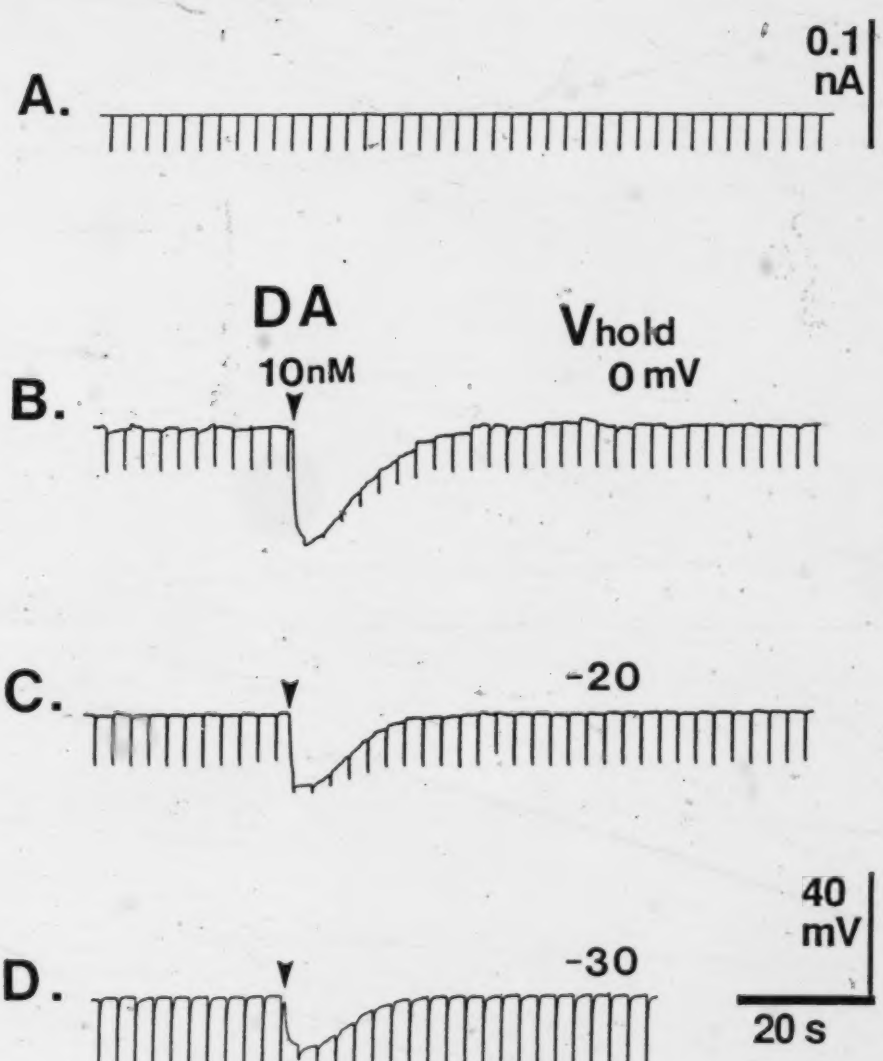
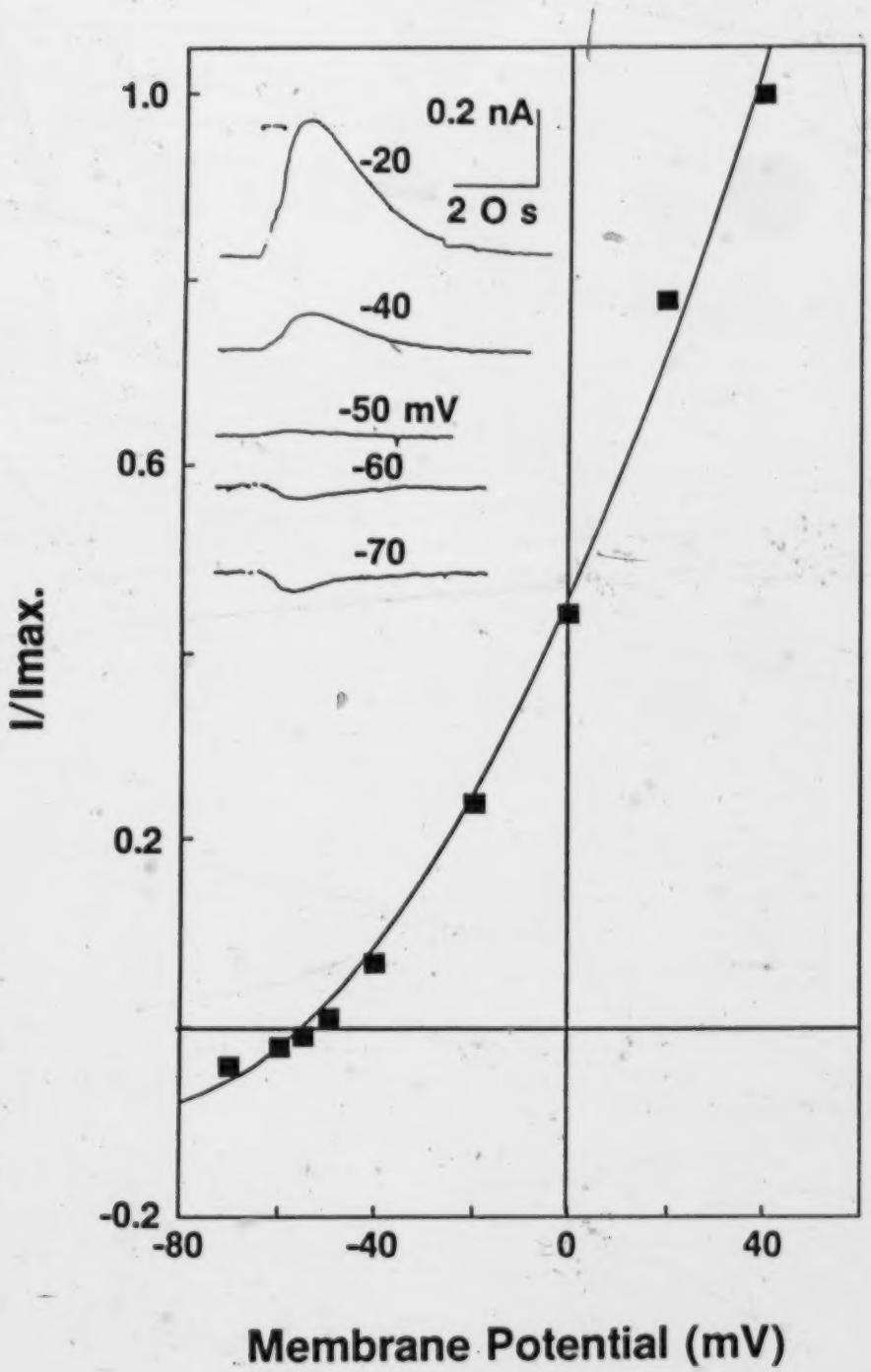

A.
0.1
nA
DA
10nM
Vhold
0 mV
B.
C.
-20
D.
-30
40
mV
20 s

Figure III-9. Voltage-dependence of the dopamine-induced outward current.

Dopamir.e was applied at 0.1 mM. Each application was given at a different membrane holding potential, consisting of 5 consecutive ejections of 900 ms each at 138 KPa. The inset shows selected current responses close to the reversal potential of –53mV.


1.0
0.6
0.2
-0.2
I/Imax.
0.2 nA
2 O s
-20
-40
-50 mV
-60
-70
-80
-40
0
40
Membrane Potential (mV)

current-voltage plots at –60 mV and 0 mV, increased by a factor of 5.1 ± 0.8 (Mean ± SEM, n=8 ; range from 2.2 to 8.8) as the membrane potential was depolarized. In some cases, the current-voltage relation of responses was of nearly zero slope conductance over the membrane potential range –40 to –60 mV. When neurons were depolarized beyond –20 mV the slope conductance increased rapidly.

Changes in the slope of the current-voltage relationship highlight the voltage sensitivity of the response, but reveal little about the underlying conductance mechanism. For a parallel conductance model (Ginsborg, 1967), the agonist-evoked current I_x is related to the agonist-activated ionic (chord) conductance G_x by the equation:

$$I_x = G_x (E_m - E_x)$$

where E_m is the membrane potential and E_x the reversal potential of the agonist-activated current. I was able to record dopamine-evoked currents on both sides of the reversal potential in many cases and could therefore estimate the driving force (E_m - E_x) either by direct measurement or from the intersection of the current-voltage curve on the voltage axis. The dopamine-activated ionic conductance was then calculated and plotted versus the membrane potential (Fig. III-10A). The conductance activated by dopamine was an outward rectifier, and increased as the membrane was depolarized. The outward rectification ratio obtained by dividing the chord conductance at +40 mV by the chord conductance at –40 mV was around four. The continuous line through the closed circles in Fig. III-10B is obtained by the equation showing the Boltzmann relation:

$$G/G_{max.} = [1 + \exp\{(V - V_h)/k\}]^{-1}$$

where V and V_h represent the membrane potential and the potential for half-maximal conductance and k describes the slope of the curve. The average values for V_h and k obtained from 6 cells were –12.6 ± 2.6 mV (range from –24.5 to –6.1 mV) and –24.2 ± 1.5 mV (range from –30.0 to –20.4 mV), respectively.

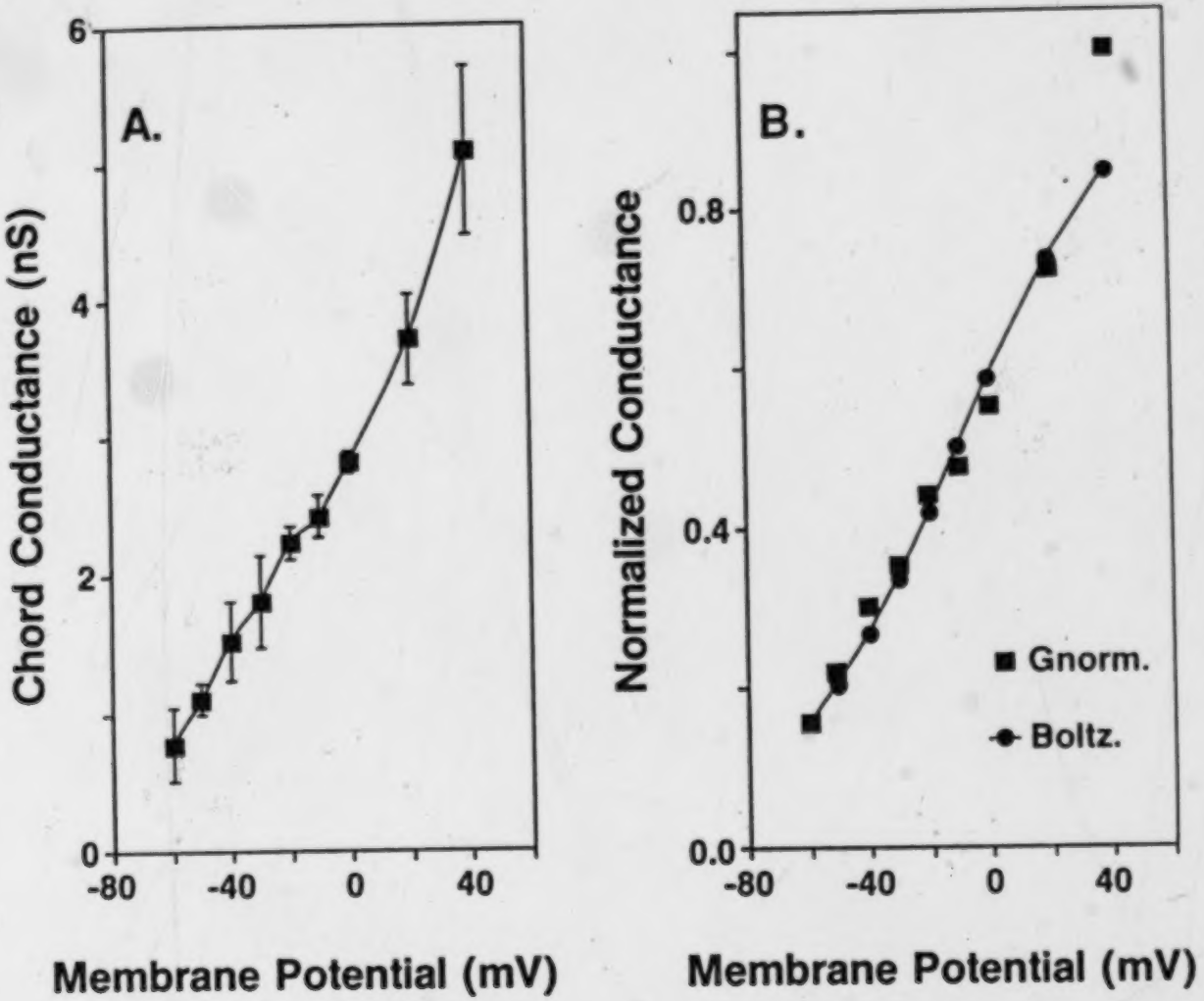
E. Ionic dependence of the inhibitory current.

The reversal potential of the dopamine-evoked response suggested that the inhibitory effects evoked by dopamine were caused by an increased permeability to potassium. To determine the underlying ionic mechanism of the response, the effects of ionic

Figure III-10. Chord conductance-membrane potential relationship of the dopamine response.

A. Chord conductances were calculated by dividing the peak current amplitude at a given membrane potential by the driving force for dopamine-activated ionic current. The conductance mechanism activated by dopamine behaves as an outward rectifier, and increases in amplitude as the membrane is depolarized.

B. The relationship of the normalized conductances (■) to membrane potential follows the Boltzman function (●), indicating the voltage sensitivity of the dopamine response. The line through ● is the best-fit curve obtained by the "simplex" method (Nelder and Mead, 1965) to the equation given in the text. The values for V_h and k were –10.2 and –29.3 mV, respectively. A k of –29.3 implies an e-fold change in conductance for a potential change of 29 mV.


A.
Chord Conductance (nS)
6
4
2
0
-80
-40
0
40
Membrane Potential (mV)
B.
Normalized Conductance
0.8
0.4
0.0
Gnorm.
Boltz.
-80
-40
0
40
Membrane Potential (mV)

substitutions on the reversal potential and the amplitude of the dopamine response were examined.

Figure III-11A & B show that the reversal potential of the outward current was not altered by changing the sodium (n=5) or chloride (n=3) equilibrium potentials, respectively. However, the amplitude of the current induced by dopamine declined when the external chloride ion concentration was reduced from 510 mM to 77 mM (Fig. III-11B). This reduction in the amplitude of the response was not reversible. Neurons did not recover their responsiveness to dopamine even after changing the bath solution from low-Cl^--saline to NASW. It is unlikely that this reduction of the current was due to desensitization of receptors or run-down of the cell since this effect was not seen in both Na and K ionic substitution experiments. It is clear that the reversal potential of the dopamine response is insensitive to changes in extracellular sodium and chloride ion concentration. The dopamine inhibitory response reversed around –55 mV which is close to the calculated equilibrium potential for potassium (–59.1 mV). Thus, it appeared likely that the dopamine effect was mostly due to an increase in potassium permeability. The dopamine reversal potential followed the equilibrium potential for potassium when the concentration of extracellular potassium ion was varied. Figure III-12A illustrates the effect of an increase in extracellular potassium ion concentration from 13.4 to 55.4 and 105 mM. The reversal potential of the dopamine response decreased from –55.0 to –27.2 and –6.6 mV (n=3) respectively, the values of which approximated those predicted by the Nernst equation (Fig. III-12B). The amplitude of the response also decreased as the concentration of extracellular potassium ion increased, which could be explained by the decreased driving force.

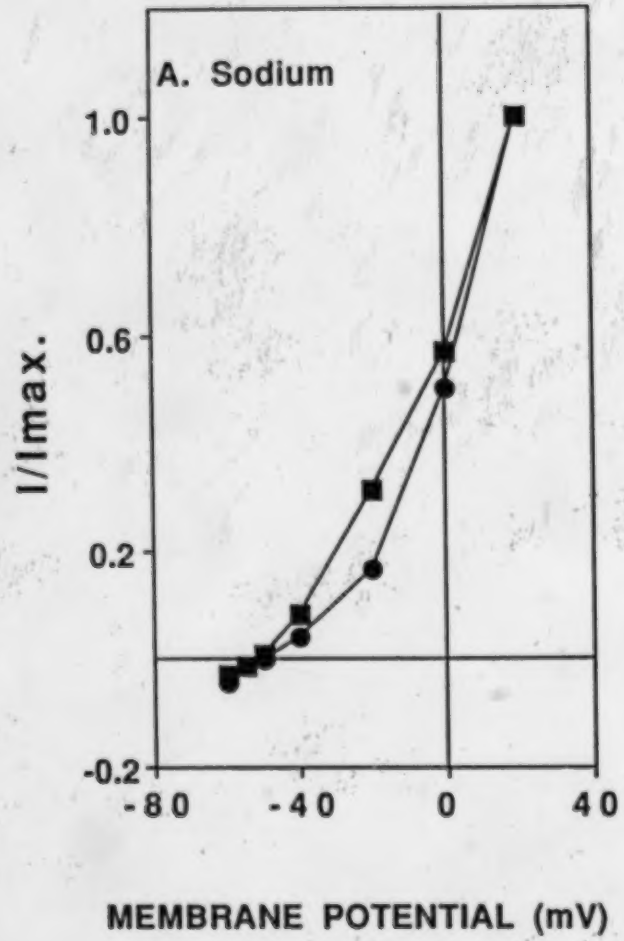
F. Effect of dopamine on the voltage-gated K^+ current.

When SMNs were continuously perfused with saline in which sodium and calcium were replaced with choline, step depolarizations produced a large outward current (5-10 nA) carried by potassium ions. To minimize the error due to series resistance, the following precautions were taken: 1) microelectrodes of less than 1.2 MΩ tip resistance were used. 2) the concentration gradient of potassium ions across the membrane was reduced fourfold by increasing the potassium extracellular concentration from 13.4 to

Figure III-11. Ionic dependence of the dopamine response.

A. Current-voltage plot showing the effect of sodium ion substitution on the dopamine response. Changing the bath solution from NASW (■) to Na-free saline (●) did not cause any significant changes in either reversal potentials or amplitudes of dopamine responses.

B. Current-voltage plot showing the effect of chloride ion substitution on the dopamine response. Dopamine responses were obtained from a SMN under continuous perfusion with NASW (■), low-Cl (●), and NASW (▲). In all cases the reversal potentials remained constant. However, the amplitude of the responses were reduced after ionic substitution and did not recover their amplitudes after washing with NASW.


A. Sodium
1.0
0.6
0.2
-0.2
I/Imax.
-80
-40
0
40
MEMBRANE POTENTIAL (mV)

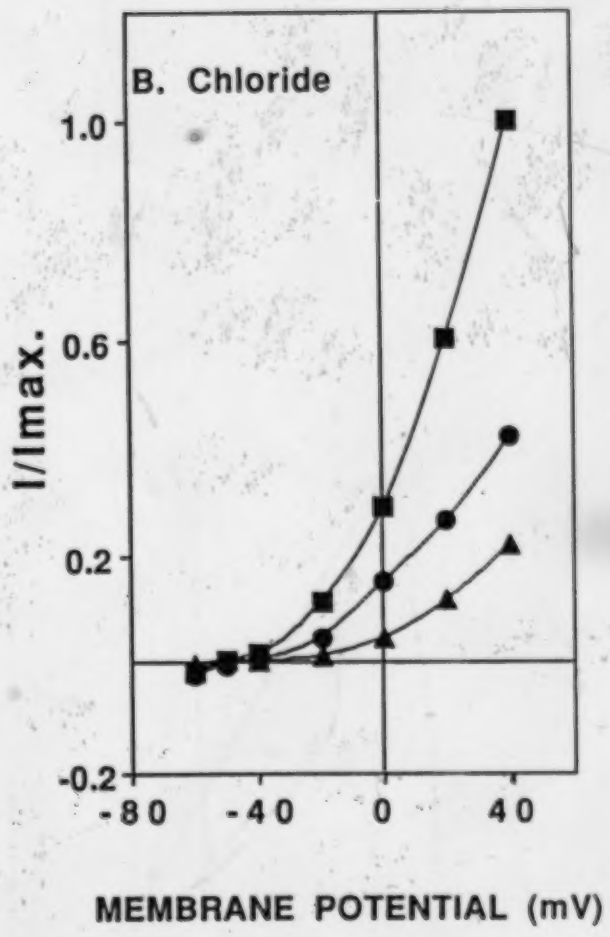
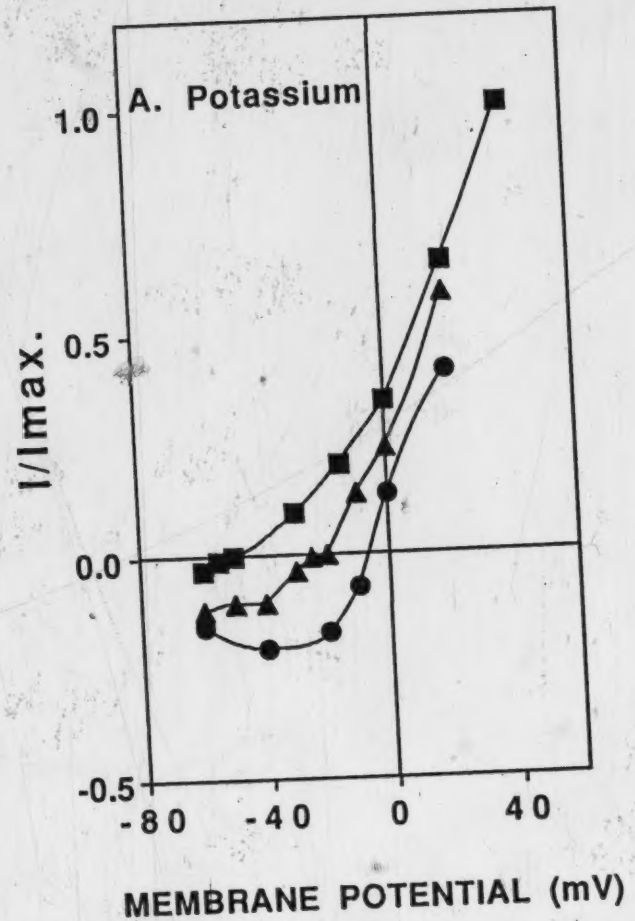

B. Chloride
1.0
0.6
0.2
-0.2
I/Imax.
-80
-40
0
40
MEMBRANE POTENTIAL (mV)

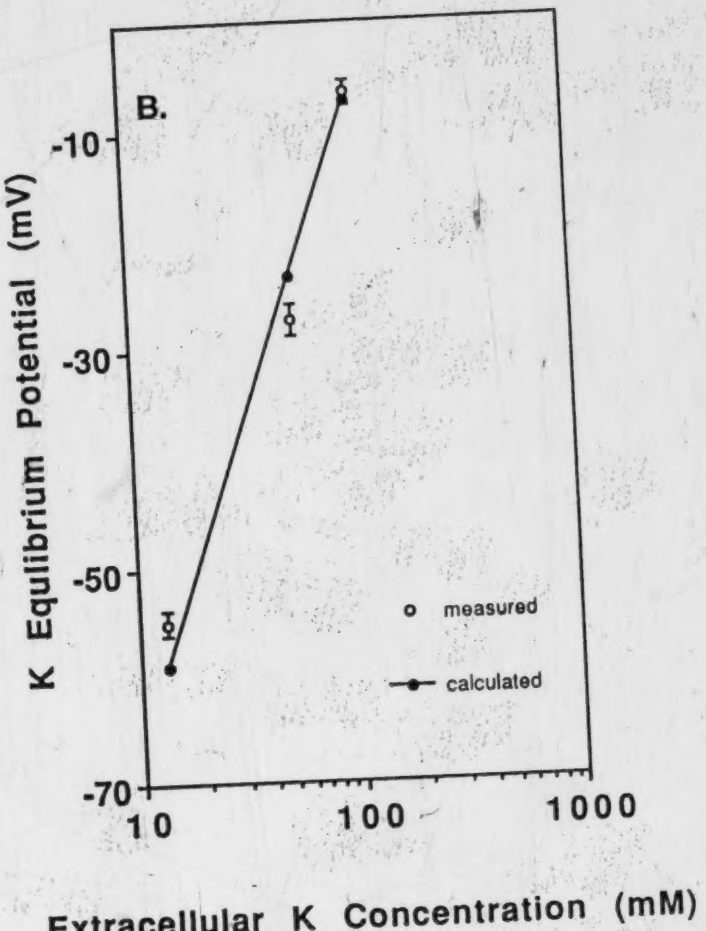
Figure III-12. The potassium dependence of the dopamine response.

A. Current-voltage plot showing the effect of potassium ion substitution on the dopamine response. Dopamine responses were obtained from a SMN in continuous perfusion with NASW (13.4 mM, ■), HHK (105 mM, ●), and HK (55.4 mM, ▲). As the extracellular K^+ concentration increased, the reversal potential of the dopamine response decreased so that it approximated the K^+ equilibrium potential. The decreasing amplitude of the response could not be due to run-down of the cell since the dopamine responses increased when the K^+ concentration of the perfusion solution decreased from HHK (105) to HK (55.4).

B. The relationship between the reversal potential of the dopamine response at different extracellular potassium concentrations. The measured reversal potentials (○) are given as mean reversal potential ± SD (N=4). A curve was calculated which fits the Nernst equation for the single ion (potassium).

T = 293K.


A. Potassium
1.0
0.5
0.0
-0.5
I/Imax.
-80
-40
0
40
MEMBRANE POTENTIAL (mV)


B.
-10
-30
-50
-70
K Equlibrium Potential (mV)
measured
calculated
10
100
1000
Extracellular K Concentration (mM)

55.4 mM, resulting in a reduction in the amplitude of the voltage-gated potassium currents.

Figure III-13A shows typical outward currents elicited from SMNs in the absence of dopamine when step pulses were applied in 10 mV increments from a holding potential of –40 mV. The small inward currents that were recorded under control conditions (A) might be due to residual sodium ions, indicating that the perfusion was not complete even after 15 min. In later experiments (Fig. III-14; see also Chapter IV), especially when pharmacological agents were used, a fast perfusion system was employed which removed the inward current. When 1 μM of dopamine solution, in sodium- and calcium-free saline, was continuously delivered to the cell using a Picospritzer, the inward currents were obliterated and the outward currents increased significantly (Fig. III-13B). The inward currents may have been swamped by the increased K^+ current. Panel C shows the outward currents obtained 12 min after the end of dopamine application. The completion of dopamine application was immediately followed by perfusion of the bath with sodium and calcium-free saline. The outward currents in panel D were obtained by subtracting the control (panel A) from the traces in the presence of dopamine (panel B). Panel D apparently suggests that at least two different types of outward currents were increased by dopamine. However, these traces may also have included the inward currents which could not be totally removed in the control. The current traces in panel E obtained by subtracting the control (panel A) from the wash traces (panel C) represent the inward currents which should have been washed out in the control experiment. Thus, panel F shows that dopamine enhanced at least two kinetically distinct K currents, possibly a fast inactivating current (I_A-like) and a rectifier-like current (I_K-like). I have called these currents I_a and I_b, respectively.

Considering the incomplete perfusion as shown in panel A, it is possible that dopamine was not completely washed out in panel C, even though the cell was continuously perfused with sodium- and calcium-free saline. Therefore, the current traces in panel E may show not only the inward currents in the control but also an outward current induced by residual dopamine molecules which were not washed away. It is also possible that two different subtypes of dopamine receptors, such as high and low-affinity, are present in SMNs; that is, dopamine may evoke an outward current as

Figure III-13. Dopamine enhances voltage-dependent K currents.

Voltage-dependent K currents were measured in a SMN. Step depolarizing pulses of –30 to 50 mV in 10 mV increments, 450 ms in duration, were applied from a holding potential of –40 mV. Dopamine (1 μM) was applied for 6 min. using a Picospritzer (pressure 138 KPa) and immediately followed by a wash. Note that dopamine appears to increase the slowly inactivating current more than the transient current.

A. Potassium currents recorded under control conditions.

B. External application of 1 μM dopamine resulted in a marked increase of a K current.

C. The dopamine effect was partially removed by washing.

D. Currents obtained by subtracting the control from the dopamine-treated traces.

E. Currents obtained by subtracting the control from the washed traces.

F. Currents obtained by subtracting the washed from the dopamine-treated traces, showing that at least two different K channels were probably activated by dopamine.

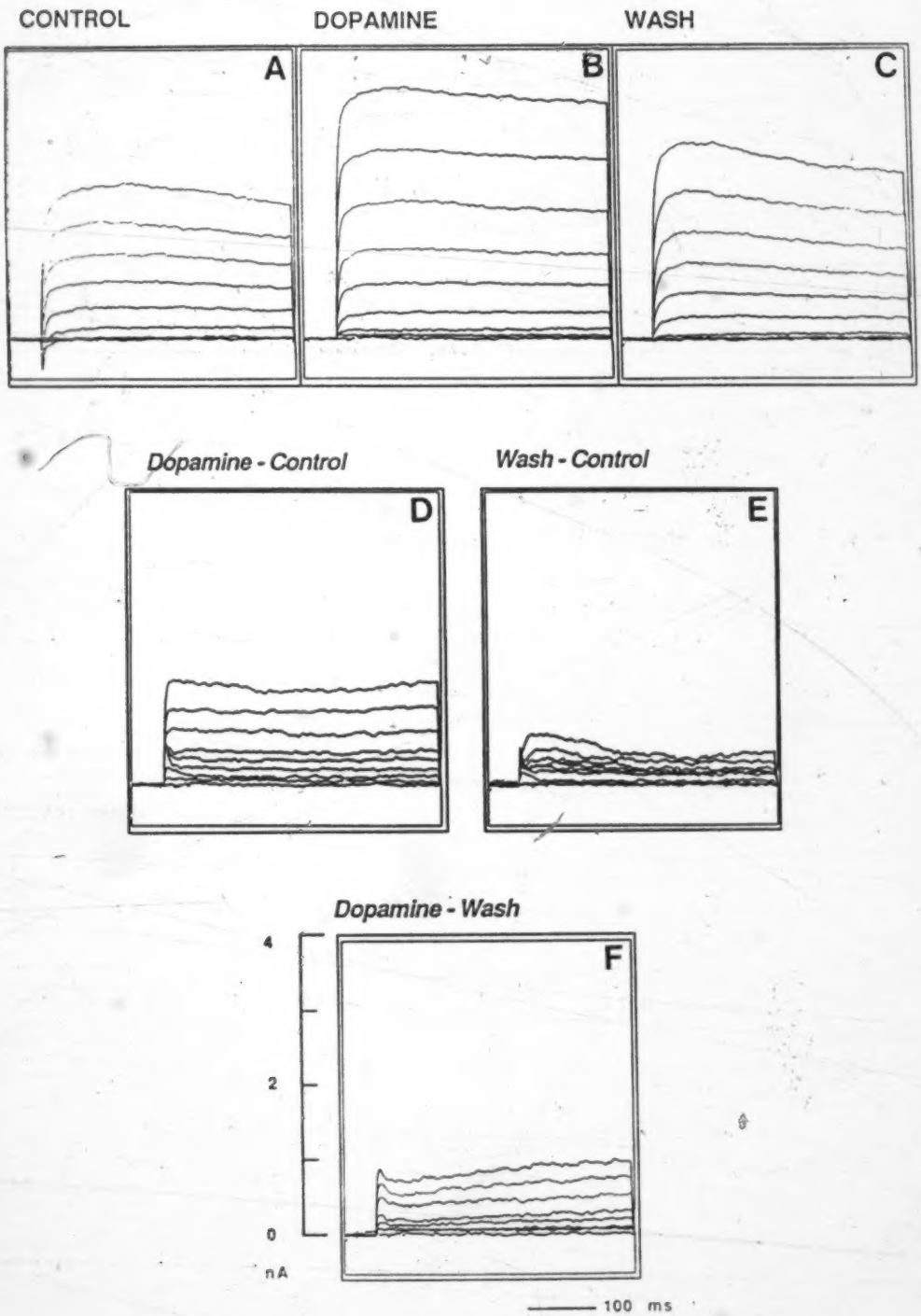
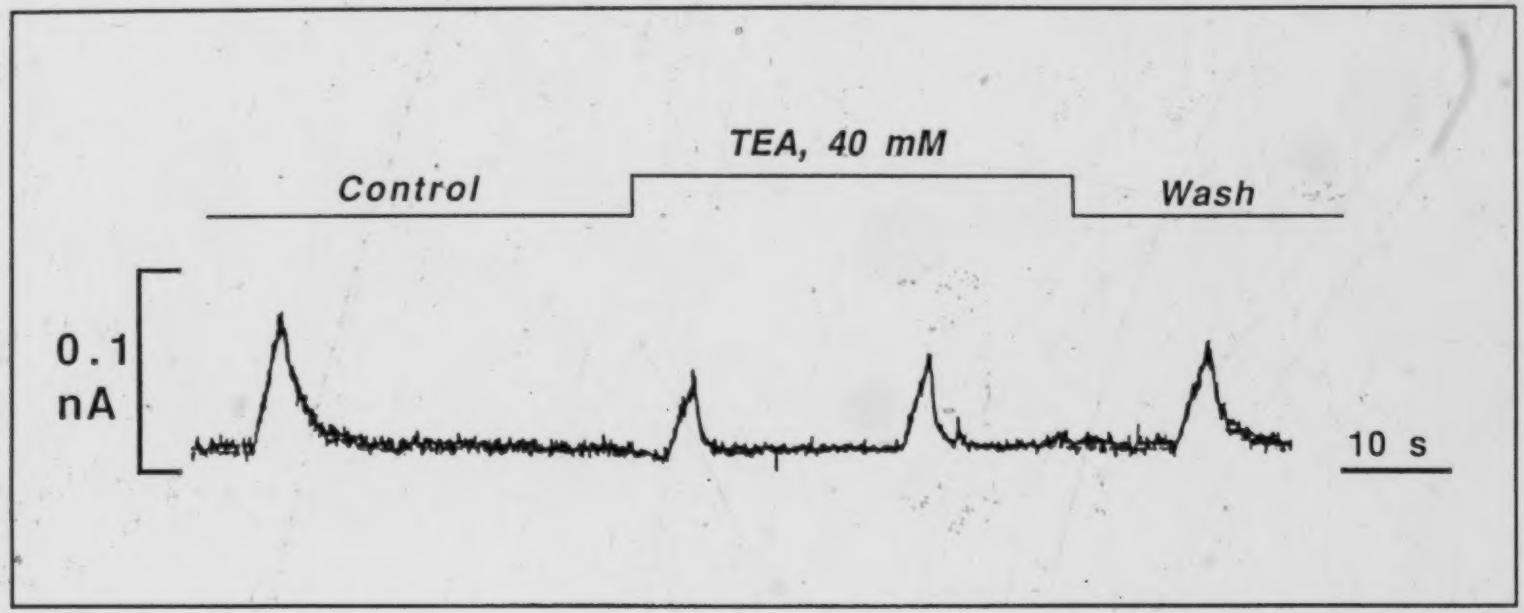

CONTROL
DOPAMINE
WASH
A
B
C
Dopamine - Control
Wash - Control
D
E
Dopamine - Wash
F
4
2
0
nA
100 ms

Figure III-14. TEA reduces the dopamine-induced outward currents.

One μM dopamine applied for 2s using a Picospritzer (pressure 138 KPa) evoked an outward current from a swimming motor neuron which was held at –20 mV. When the neuron was exposed to 40 mM tetraethylammonium chloride (TEA) solution using a rapid exchange perfusion system (See Chapter IV), the amplitudes of the dopamine-induced outward currents were reduced by 40%.


TEA, 40 mM
Control
Wash
0.1
nA
10 s

shown in panel E through a high-affinity dopamine receptor and outward currents as shown in panel F through a low-affinity dopamine receptor. Separation of the different outward currents, either pharmacologically or kinetically, were not attempted in this study. Further experiments are needed.

A portion of the dopamine-induced current was sensitive to tetraethylammonium (TEA). Figure III-14 shows that dopamine-induced currents from a SMN were reduced drastically by 40 mM TEA. Although many voltage-dependent K currents are TEA-sensitive (Hille, 1984), it does not necessarily imply that TEA-sensitive currents are voltage-dependent, though this is likely.

G. Localization of the putative dopamine receptors.

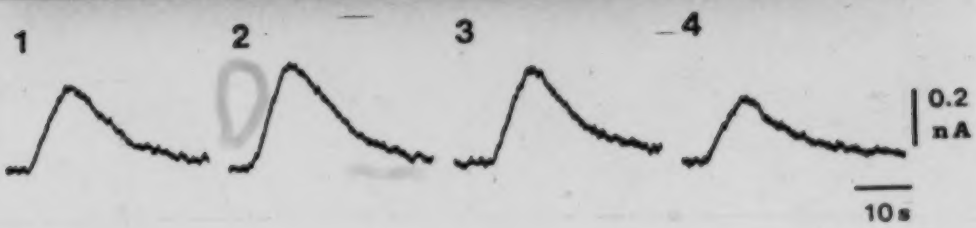
There are no obvious experimental data showing that the dopamine-receptors are localized to specific areas of the SMN, although some cells gave larger currents when dopamine was applied to processes rather than the soma (N=2). For example, in Fig. III-15, neurite (2) gave a 20% larger response than the soma (1) to applied dopamine.

Since the pipette delivering dopamine was positioned close to the cell, the concentration of dopamine was significantly greater at the cell surface immediately under the pipette than anywhere else on the cell. This was confirmed by adding a phenol red solution to the pipette when a distinct ejection plume was seen with little diffusion or mixing.

Figure III-15. Distribution of dopamine receptors.

Ten μM of dopamine was applied for 5s using a Picospritzer (pressure 138 KPa) successively to the base (1), the tip (2), the middle (3) of a process, and the centre of a soma (4) of a swimming motor neuron. The arrows point in the direction that the opening of the micropipette which delivered dopamine was facing. During the experiment, the cell was continuously perfused with normal artificial sea water. The photograph was taken before recording. Holding potential, –20mV; *re*, the position of a recording electrode.

re
2
3
4
1


1
2
3
4
0.2
nA
10s

DISCUSSION

Although there is considerable electrophysiological evidence that chemical neurotransmission in the Cnidaria (Anderson, 1985; Mackie & Meech, 1985; Satterlie, 1984; Spencer, 1982) is similar to that seen in higher phyla (such as annelids, molluscs, arthropods and chordates), there have been no cellular studies which give definitive data on the mechanisms of a transmitter's actions. Similarly there is only fragmented information on the nature of the transmitters (Martin & Spencer, 1983). Such information is needed for reconstructing the early evolution of interneuronal chemical communication, since it is presumed that it was in this group, or a common ancestor, that such mechanisms first evolved. In this study I show that dopamine is neuroactive in a class of neurons. Namely, dopamine gates an increase in potassium conductance that leads to a transitory hyperpolarization and inhibition of spontaneous spiking in SMNs.

Inhibitory actions of dopamine

The current clamp experiments showed that brief (2s to 4s) pulses of dopamine at 10 µM had an inhibitory effect on SMNs by eliciting immediate hyperpolarizations of 5 to 15 mV which lasted as long as 15-30s. These hyperpolarizations were sufficient to either slow or arrest spiking produced by anodal-break stimulation (Figs. III-5A & B). The threshold concentration for producing hyperpolarizations has not been precisely determined, however voltage-clamp data showed that dopamine at 10^{-8} M elicited an outward current at a holding potential of –20 mV. Dopamine also had an inhibitory effect on SMNs by decreasing the input resistance (Figs. III-6 & -7) and thus shunting other synaptic responses (Zucker, 1987).

The membrane hyperpolarizations seen after application of dopamine were shown to be due to a conductance increase which was voltage-dependent. Dopamine responses associated with a conductance increase have been reported in several other preparations, such as rat lactotrophs (Israel et al., 1987; Castelletti et al., 1989), human prolactinoma cells (Israel et al., 1985), and identified neurons of the snail *Helix aspersa* (Bokisch & Walker, 1986 ; Cox & Walker, 1988). Dopamine caused a larger

decrease in membrane resistance at more depolarized membrane potentials (Fig. III-8). The voltage-sensitivity of the 'dopamine response' could be clearly seen in voltage-clamp recordings where dopamine elicited outward currents that increased with membrane depolarization (Fig. III-9). The slope conductance increased progressively as the membrane was depolarized. Such rectifying properties of current-voltage curves have been reported in the identified *Helix* neurons (Cox & Walker, 1988) while rectification was not seen in the growth hormone producing cells of the freshwater snail *Lymnaea stagnalis* (De Vlieger et al., 1986). The dopamine-activated chord conductance also increased with membrane depolarization (Fig. III-10A). The measured rectification ratio is 4. The normalized conductance followed the Boltzman relation, also indicating that the dopamine response is voltage dependent (Fig. III-10B).

The reversal potential for this outward current was approximately –55 mV which was close to the calculated E_K of –59 mV, and since altering the potassium gradient shifted the reversal potential in a Nernstian manner (Fig. III-12), it is concluded that the dopamine-induced current is carried by potassium ions. Such a conclusion was further supported by the results of the ionic substitution experiments for sodium and chloride ions (Fig. III-11). The fact that the agonist responses were reduced in low-Cl^--saline may imply that there is a reduction in the driving force on potassium ions because of a loss of intracellular potassium ions due to the contribution of a Donnan equilibrium (Boyle & Conway, 1941). However, this cannot explain why the cells (n=3) did not recover their responsiveness to dopamine even 30 min after replacement of low-Cl^--saline with NASW. It is likely that the WCR configuration lessens the importance of a Donnan equilibrium of the type described by Boyle and Conway since the intracellular ionic concentrations of these ions should remain constant during an experiment.

The exact nature of the channels responsible for the dopamine-induced potassium current is unclear. There are several ways to explain the underlying mechanism of dopamine responses related to the channel properties. *First*, dopamine may increase the conductance of ligand-gated potassium channels, resulting in membrane hyperpolarization. Membrane hyperpolarization may also remove steady-state inactivation of a purely voltage activated potassium channel, resulting in a decrease of spike duration (Fig. III-5C). For example, in current-clamp mode the duration of

action potentials varies with the membrane holding potential. Spencer et al. (1989) suggested that unmasking of a fast, transient potassium current ($I_{K\text{-}fast}$) at hyperpolarized holding potentials might be responsible for duration decreases. *Second,* the fact that action potential duration was decreased during exposure to dopamine as shown in Fig. III-5C could also suggest modulation of voltage-sensitive currents. In fact, dopamine affected voltage-dependent potassium channels in SMNs, generating at least two different currents I_a and I_b (Fig. III-13) which have similar properties to the transient (I_A) and delayed rectifying (I_K) potassium currents described in many organisms. This is not surprising. Dopamine increases delayed rectifying (I_K) and transient (I_A) potassium currents in rat lactotroph cells (Lledo et al., 1989) and I_A current in the MMQ clonal pituitary cells (Login et al., 1990). Dopamine's action on voltage-dependent potassium channels may be direct or indirect via a second messenger system. *Third,* it is possible that voltage-sensitive, ligand-gated potassium channels may be present in jellyfish neurons.

Modulation of potassium channels would change the pattern of cell depolarization and thus alter the Ca^{2+} flux through voltage-gated calcium channels. There is also an influx of Ca^{2+} with each action potential in SMNs (Spencer et al., 1989). Therefore, two membrane events, in the presence of dopamine, hyperpolarization and the absence of action potentials, may have a cumulative effect in decreasing the concentration of intracellular Ca^{2+} ions. In addition, catecholamines have been shown to decrease the duration of Ca^{2+} spikes in several invertebrate and vertebrate neurons. For example, in rat sympathetic neurons norepinephrine decreases the duration of the Ca^{2+} spike (Galvan & Adams, 1982) and similar effects were observed in cultured chick embryo dorsal root sensory neurons after application of several transmitters including dopamine (Dunlap & Fishbach, 1980; Canfield & Dunlap, 1984). In both cases catecholamines decreased the Ca^{2+} conductance. Marchetti et al. (1986) demonstrated that dopamine and norepinephrine reversibly reduced calcium channel activity in outside-out membrane patches of cultured sensory and sympathetic neurons of the chick. Dopamine-induced Ca^{2+} conductance decreases have also been reported for identified neurons in the snail *Helix aspersa* (Paupardin-Tritsch et al., 1985). Although it is unknown whether catecholamines can directly modulate Ca^{2+} conductances in jellyfish

neurons, it is possible that the intracellular level of Ca^{2+} may be indirectly influenced by dopamine through its effect on potassium conductances.

Does applied dopamine mimic IPSPs seen *in vivo*?

Two rather different sources of inhibitory input to SMNs have been identified *in vivo*. In the first, photoreceptive oscillatory or 'O' neurons, which descend into the nerve-rings from the ocelli, appear to make inhibitory synapses onto the SMN network. Shadowing the ocelli produces a graded hyperpolarization and inhibition of spontaneous membrane potential oscillations in the 'O' neurons. Since these hyperpolarizations are followed instantaneously by depolarizations of the SMNs, it was concluded that 'O' neurons tonically release an inhibitory transmitter onto SMNs (Arkett & Spencer, 1986a, b) and that the depolarization and consequent spiking of SMNs when jellyfish are shadowed is due to release from tonic inhibition. In this study a 'rebound' depolarization following the application of dopamine has not been recorded. Therefore it seems unlikely that dopamine is the substance responsible for the shadow response of SMNs. Also the rapid desensitization (refer to the Chapter IV) of the putative dopamine receptor in a SMN implies that dopamine would not be a tonically released substance.

A second type of IPSP can be recorded spontaneously by intracellular microelectrodes from the SMN network. These IPSPs are associated with action potentials propagated in the surrounding epithelium (Spencer, 1981), and were assumed (Mackie, 1975, in the case of *Stomatoca*) to result from an electric field effect or a temporary change in the concentration of an extracellular ion. The evidence for supposing that these IPSPs are not chemically mediated is that blockade does not occur with a 3:1 mixture of sea water and isotonic magnesium chloride solution. Nevertheless it is possible that had higher concentrations of Mg^{2+} anaesthesia been used blockade would have been achieved. A 1:1 solution was needed to block the shadow response of SMNs (Arkett & Spencer, 1986a). Even so, some chemical synapses in hydrozoans are known to be insensitive to Mg^{2+} (Kerfoot et al., 1985). Thus it is possible that the IPSPs seen in SMNs during "crumpling" behavior (King & Spencer, 1981) are due to the actions of an inhibitory transmitter. The time courses of the spontaneous IPSPs recorded *in vivo* and those recorded after application of dopamine *in vitro* are both slow and sustained. Thus of the two types of IPSP seen *in vivo*, that associated with

epithelial impulses is more consistent with a mechanism involving mediation by dopamine. King and Spencer (1981) showed that neurons were involved in transferring epithelial excitation to the effector muscles responsible for 'crumpling' and thus these same neurons, rather than the epithelial cells, might be responsible for releasing an inhibitory transmitter. This group of neurons has not yet been identified. The comparative sensitivities of these two synaptic events to Mg^{2+} needs to be examined more carefully.

If the findings described here are considered in combination with the prior observation that dopamine is present in the nerve-rings of this jellyfish (Chapter II), it is attractive to propose that dopamine could be an inhibitory neurotransmitter or modulator in the central nervous system of hydromedusae.

Some of the data presented in this chapter were published in 1991 by Chung JM & Spencer AN in "Dopamine as a neuroactive substance in the jellyfish Polyorchis penicillatus." J. exp. Biol. 156: 433-451.

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CHAPTER IV.
INHIBITORY ACTION OF DOPAMINE ON A JELLYFISH NEURON VIA A D_2-LIKE RECEPTOR .

The only basic assumption involved when discussing drug action in terms of receptors is that the drugs combine in some way with the tissue they affect. In order to develop a system of thought about drug action it is necessary, in the absence of intimate knowledge, to make additional assumptions. It is important, however, that such assumptions should be made explicitly so that they may be tested where possible. This, unfortunately, is not often done. (.....omitted.....) Implicit and erroneous assumptions may be concealed in the ideas which have been expressed in this paper. If so, confusion may result and the conclusions drawn may be wrong. But, if the basic assumption is correct, it will be profitable to continue the argument.

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INTRODUCTION

The previous chapter has shown that dopamine has an inhibitory action on cultured swimming motor neurons (SMNs) of *Polyorchis penicillatus*. Applied dopamine caused membrane hyperpolarization due to increased potassium conductance. This resulted in total inhibition or reduction of spiking frequency after anodal break excitation. Inhibition was associated with decreased spike duration. These observations on the physiological actions of dopamine combined with an HPLC, GCMS spectrometric study (see Chapter II) which demonstrated the presence of DA in nerve-rich tissues of *P. penicillatus*, suggest that dopamine acts as a neurotransmitter and/or neuromodulator in this jellyfish.

The present study examines whether the dopamine-induced responses are mediated through a conventional receptor molecule, and if so, to determine the degree of selectivity of the receptor. The characteristics of a receptor can be determined biochemically (binding assay) and electrophysiologically using known agonists and antagonists for dopaminergic receptors in other invertebrates and mammals. There are advantages and disadvantages to both approaches. Receptor density and affinity can be determined more accurately from binding studies, while relative efficacy of agonists can be only measured electrophysiologically; which was the technique used in this study. Ideally both approaches should be used to elucidate the properties of a receptor and the mechanisms of drug-receptor interactions.

MATERIALS AND METHODS

All experiments were performed on SMNs cultured from the bell margin of the jellyfish *Polyorchis penicillatus* as previously described (Chapter III). The recording methods and data aquisition were also identical to those described previously.

Drug preparation and method of application.

All drug working solutions were prepared daily by dilution of stock solutions using artificial sea water (pH 7.5). The stock solutions (0.1M) of amines and 3,4–dihydroxyphenylglycol (DHPG) were prepared in deionized double-distilled water. The stock solutions of spiperone (1 mM), fluphenazine (10 mM), and propranolol (10 mM) were prepared in absolute ethanol. Stock solutions (10 mM) of yohimbine, domperidone, haloperidol, and SKF 83566 were prepared in absolute DMSO. SKF 38393 (0.1 mM) and LY 17155 (1 mM) stock solutions were also prepared in DMSO. All stock solutions were frozen at –20 °C after being divided into aliquots of 500 µl.

Amines were applied to isolated cells through pipettes (methanol bubble numbers of 6.2 or 6.3) using the method described in the previous study (see Chapter III). Pulsed flow of amine solutions of 0.1 to 10s duration was obtained using a Picospritzer (General Valve Corp., Model-2) with 138 KPa applied to the pipette. Also a maintained fluid stream of amine was obtained using a Picospritzer. The amount of amine ejected was calculated by determining the ejected volume at the end of experiments as required (Sakai et al., 1979).

Antagonists were applied using a rapid delivery system similar to Johnson and Ascher's (1987). The device used for rapid solution changes consisted of five-barrelled polyethylene tubing (PE10; Intramedic no 7401; I.D, 0.28 mm). Control ASW and solutions to be tested were fed by gravity from five different reservoirs 60 cm above the microscope stage. A flow rate of about 0.6 µl/s resulted in a velocity of 10 mm/s through each tube. After the whole-cell configuration had been achieved, the gang of tubes was positioned within 200 µm of the cell with a micromanipulator so that the the effluent from barrel 1 flowed over the neuron. Changes of solution were accomplished by aligning another perfusion barrel with the neuron. Continuous perfusion was

achieved by constant aspiration of excess fluid from the opposite side of the Petri-dish.

RESULTS

A. Amine-induced currents.

Dopamine pressure-ejected onto an SMN had an inhibitory effect, and either slowed or arrested spiking generated by anodal break excitation (Fig. IV-1A; see also Chapter III). From a resting potential of –30 mV, 10 nM of dopamine produced 18 mV of hyperpolarization accompanied by a marked decrease of input resistance from 0.83 GΩ to 0.17 GΩ. Within 20s after the onset of the response, the hyperpolarized membrane potential returned to the resting value and the input resistance fully recovered. A second dopamine pulse, applied 102s after the first, produced the same effects on both membrane potental and input resistance (Fig. IV-1B). Dopamine's action was reversible and showed little desensitization at these time intervals (however see below).

Applications of dopamine at concentrations ranging from $1x10^{-8}$ to $1x10^{-3}$M generated outward currents when the membrane potential was held more positive than –55 mV, which was close to the potassium equilibrium potential (see Chapter III). Two different approaches were employed to examine the dose-response relationship. First, dopamine was applied to the cell using various pulse durations. The amount of dopamine delivered with each pulse duration was determined by measuring the diameter of the droplet of dopamine solution ejected under mineral oil (Sakai et al, 1979). Figure IV-2A shows the linear relationship between pulse duration and volume ejected and figure IV-2B shows a dose-response curve. Typical dopamine-induced outward currents from a SMN which was voltage-clamped at –20 mV are shown in Fig. IV-2C. Although the amplitude of the outward current increases with increasing ejection pulse duration, the relationship is not linear. The dose-response curve obtained from 5 different cells which were held at +20 mV (Fig. IV-2B) suggests that there is saturation of the receptor. The amounts of dopamine ejected were assumed to be proportional to dopamine concentrations impinging on target cells. However, it is difficult to determine the exact concentrations of dopamine that receptors might be exposed to. The dopamine concentration in the pipette is the upper limit of the concentration that could have reached the cell. To circumvent this problem, I also applied dopamine continuously while recording. Figure IV-3A shows that a prolonged application of 10 μM dopamine

Figure IV-1. Reproducibility of dopamine responses.

A. Ten μM of dopamine in saline was applied (shown by the arrow, ▼) for 5s through a micropipette (bubble number 6.3) using a Picospritzer (pressure 138 KPa). The first application of dopamine produced 18 mV, 16s of hyperpolarization and the second application of dopamine also produced the same amplitude and duration of hyperpolarization. The rebound action potential trains were obtained by switching the recording mode from voltage clamp to current clamp in a whole cell recording configuration; holding potental, –60 mV. No perfusion was performed during this experiment.

B. Ten nM of dopamine applied to a swimming motor neuron for 1s elicited hyperpolarization as well as a decrease of input resistance. A second application of dopamine produced the same effects. 30 pA hyperpolarizing current pulses of 200 ms were applied at a frequency of 0.35 Hz to the cell which was voltage-clamped at –30 mV. Saline was continuously perfused using the gravity-fed perfusion system.

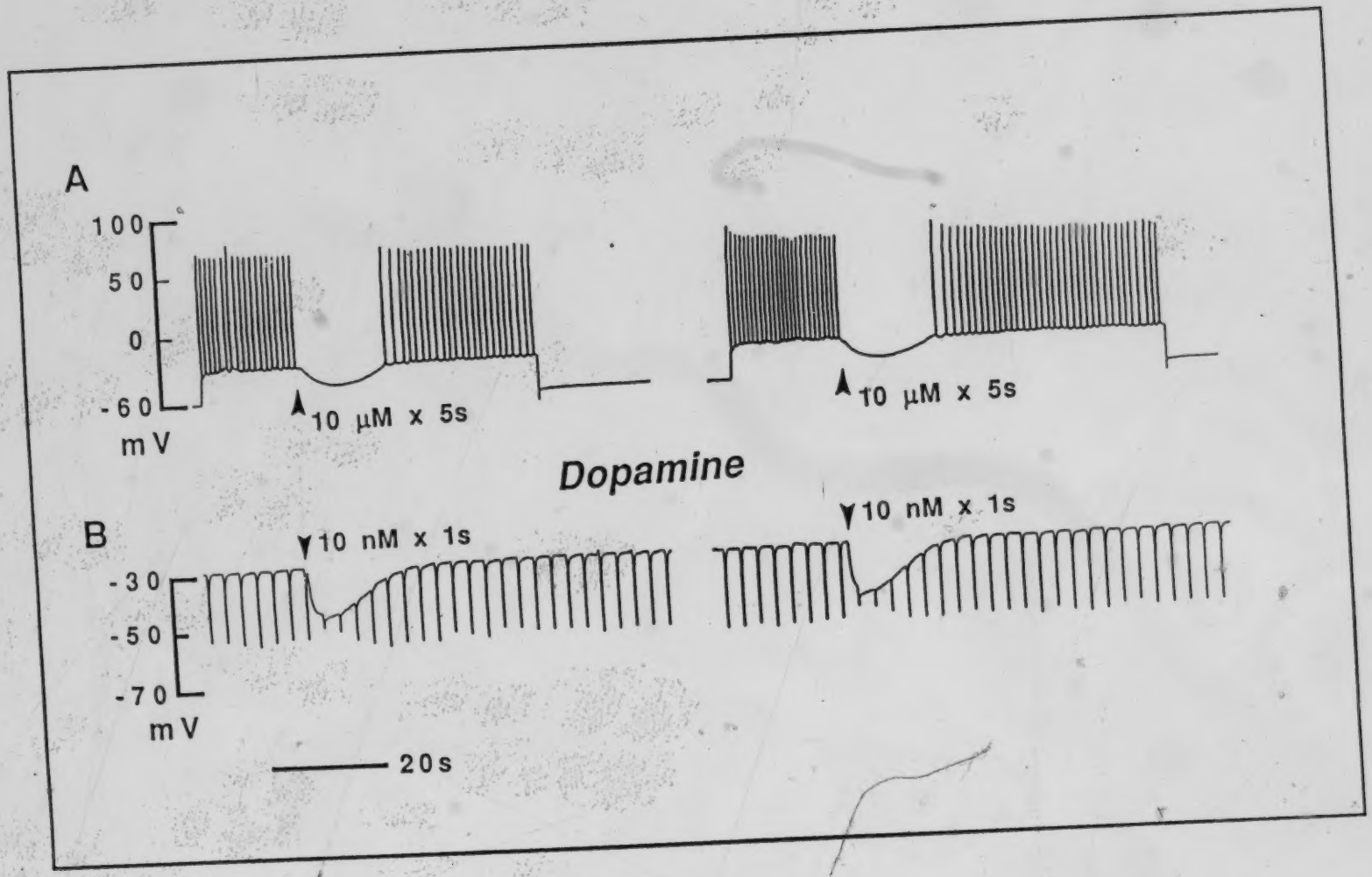
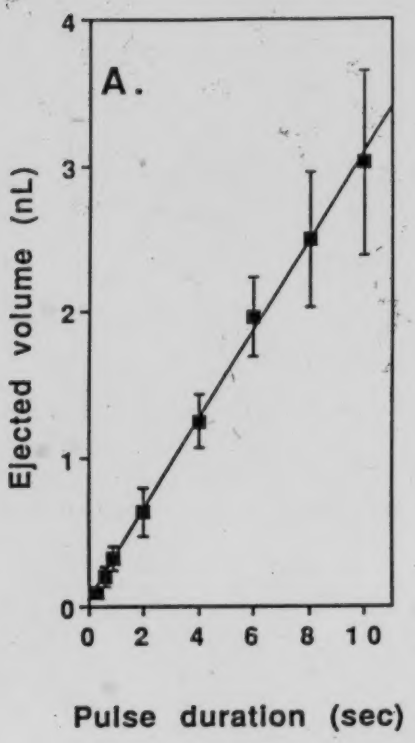

A
100
50
0
-60
mV
10 μM x 5s
10 μM x 5s
Dopamine
B
10 nM x 1s
10 nM x 1s
-30
-50
-70
mV
20s

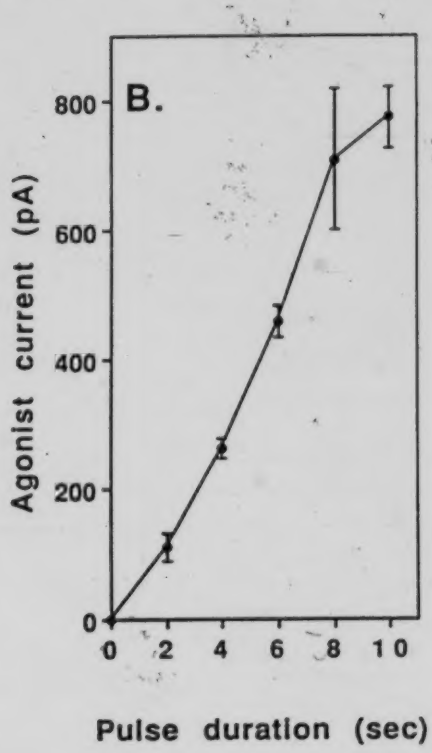
Figure IV-2. Relationship between duration of pressure ejection pulse, ejected volume of dopamine solution and agonist current.

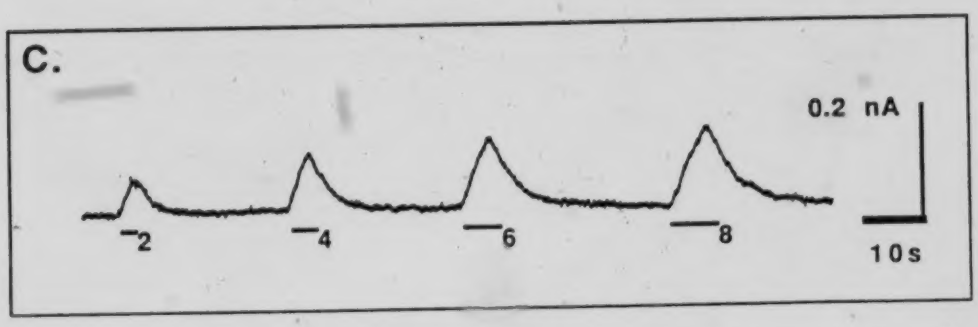
A. Relationship of the duration of the pressure pulse to the volume ejected out of the pressure pipette. One percent of phenol red solution (w/v in normal saline) was delivered into a drop of mineral oil on a micrometer slide through a micropipette (methanol bubble number, 6.2 ± 0.5 in 10 cc syringe) containing the phenol red solution, using a Picospritzer (Model II, General valve Co.; pressure, 138 KPa; room temperature, 19±1 °C). The diameter of the drop ejected was read instantaneously and the pipette lifted out of the oil. The pipette was reimmersed in a different location and the measurement repeated. The duration of the pressure-pulse was changed with a stimulator (Grass S44) connected to the Picospritzer. The sample volume ejected from the pipette changed linearly with pulse duration (r^2= 0.998). The flow rate of the solution through the picopipette was also determined at the end of the experiment by measuring the distance over which the phenol red solution moved in the pipette after a long-duration pulse. The flow rate was 0.32 ± 0.05 nl/s (N=10). This value is very close to that obtained from the curve (0.31 nl).

B. Dose-response curve of duration of ejection pulse against dopamine-induced agonist current. 10 µM of dopamine was applied to voltage-clamped motor neurons using a Picospritzer at 138 KPa with the membrane potential held at +20 mV. Pulse durations were varied under the assumption that the amounts of dopamine ejected were proportional to dopamine concentrations on the target neuron (N=5; pipette pressure, 138 KPa). A 10s pulse duration delivered 30 fmoles.

C. Pulses of 10 µM dopamine were applied at increasing durations to a swimming motor neuron which was held at –20 mV. A series of the dopamine-induced outward currents show partial saturation of the response.


A.
Ejected volume (nL)
0
1
2
3
4
0 2 4 6 8 10
Pulse duration (sec)


B.
Agonist current (pA)
0
200
400
600
800
0 2 4 6 8 10
Pulse duration (sec)


C.
0.2 nA
2
4
6
8
10 s

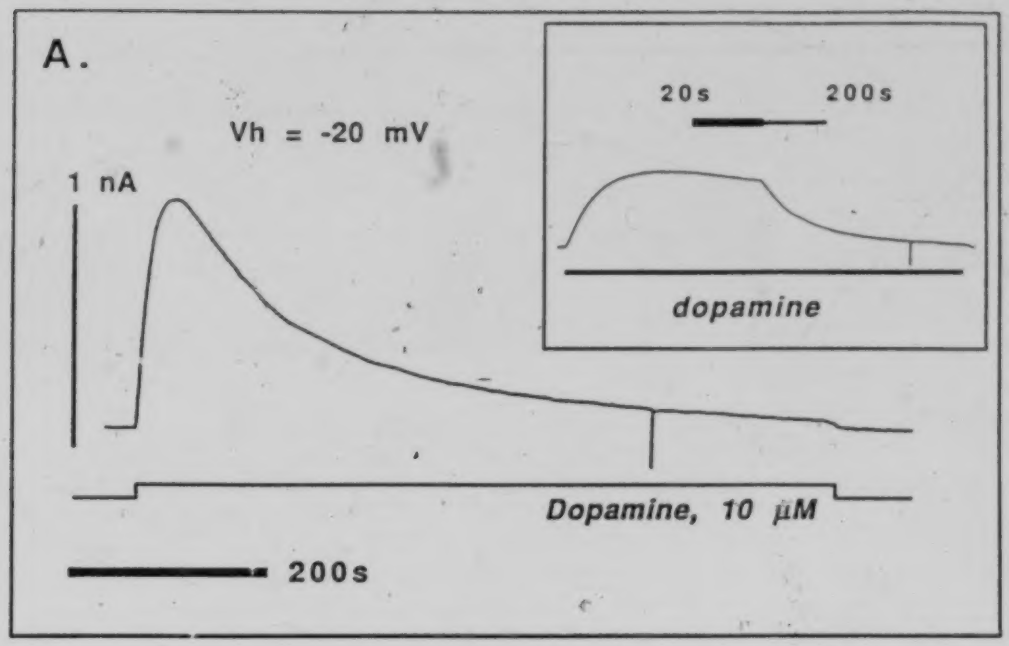
caused an outward current which was not sustained. The onset of the current was rapid (within 500 ms) while the time to peak was 30s and the time constant for the rising phase (τ_r) was 10s. This relatively slow rising phase was probably not due to mixing and diffusion of the ejected stream since these two dilution-effects should be negligible over such a short distance (50 μm). Indeed, when identical dye-filled pipettes were used at the same pressures there was no noticeable turbulence in the ejected stream. The slow rising phase of the response may represent the contribution of an intracellular biochemical cascade. On a short time scale one can recognise a plateau-phase of the response (inset of Fig. IV-3A), however on a longer time scale there is an obvious exponential decay of the response. The mean time constant for the decaying phase (τ_d) was 190s. This decay could result from desensitization of receptors and/or depletion of components involved in a cascade producing the conductance increase in potassium. Cells generated no response or a much smaller response to a second prolonged application of the same concentration of dopamine. Three different concentrations of dopamine (1 mM, 10 μM, 100 nM) were used in this study to construct concentration-response graphs. Using prolonged pulses of dopamine, outward currents were elicited in a concentration-dependent manner at a holding potential of –20 mV (Fig. IV-3B). It is interesting that 1 mM dopamine evoked much smaller responses than 10 μM dopamine.

Other monoamines also elicited outward currents, however, they were far less active than dopamine. The order of potency of these amines was determined by measuring the ratio of the amplitudes of agonist-induced currents. For example, $1x10^{-4}$ M norepinephrine applied for 5s to a SMN produced 83 pA of outward current at a V_H of 0 mV. The pressure pipette was then replaced with another that delivered the saline vehicle. The applied saline did not produce a response while application of $1x10^{-4}$ M dopamine produced 667 pA of outward current from the same cell (Fig. IV-4). The potency ratio of dopamine to norepinephrine could then be calculated; in figure IV-4 the ratio is 8.0:1 and the mean ratio from three such experiments was 8.3 : 1. The potency ratio for several amines was determined in this way; dopamine : norepinephrine and epinephrine: tyramine : octopamine : β-phenylethylamine= 580 : 70 : 13 : 3 : 1. The percentage of cells responding to each amine showed the same order as for the potency

Figure IV-3. Effects of long duration application and concentration on the dopamine response.

A. Saturation of the dopamine response. Thirty seconds after a continuous application of 10 μM dopamine using a Picospritzer (138 KPa), a dopamine-induced outward current was found to reach a maximum. The dopamine response was not sustained and decayed exponentially. The inset at a short time-scale shows saturation of the response. The cell was voltage-clamped at –20 mV and continuously perfused with a gravity-fed perfusion system.

B. Concentration-response relationship. Three different concentrations of dopamine were applied continuously using a Picospritzer (pressure, 138 KPa) to neurons held at –20 mV until saturating responses were obtained. The agonist current was measured as the peak current. Error bars represent standard errors.


A.
Vh = -20 mV
1 nA
20s
200s
dopamine
Dopamine, 10 µM
200s

B.

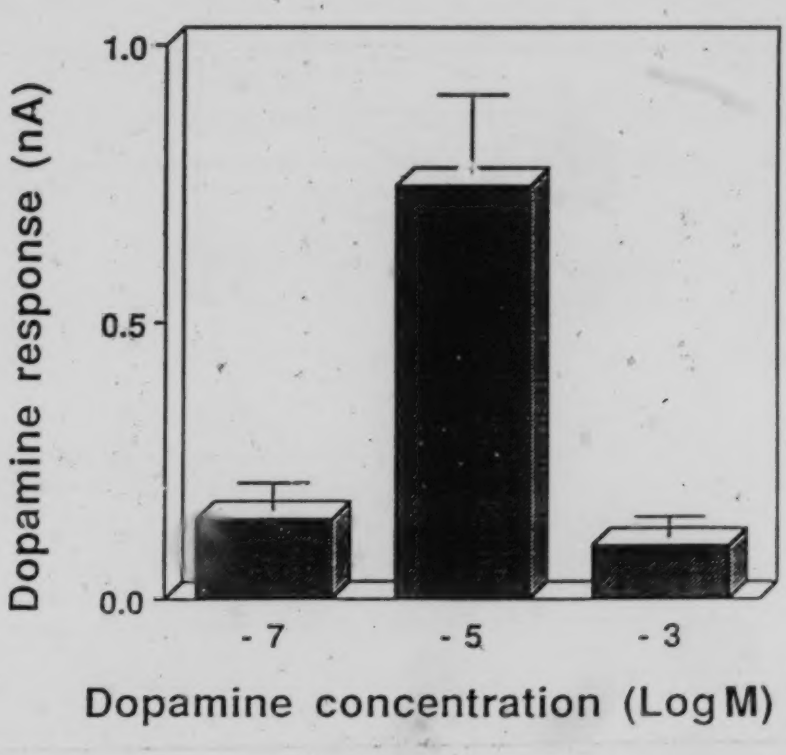
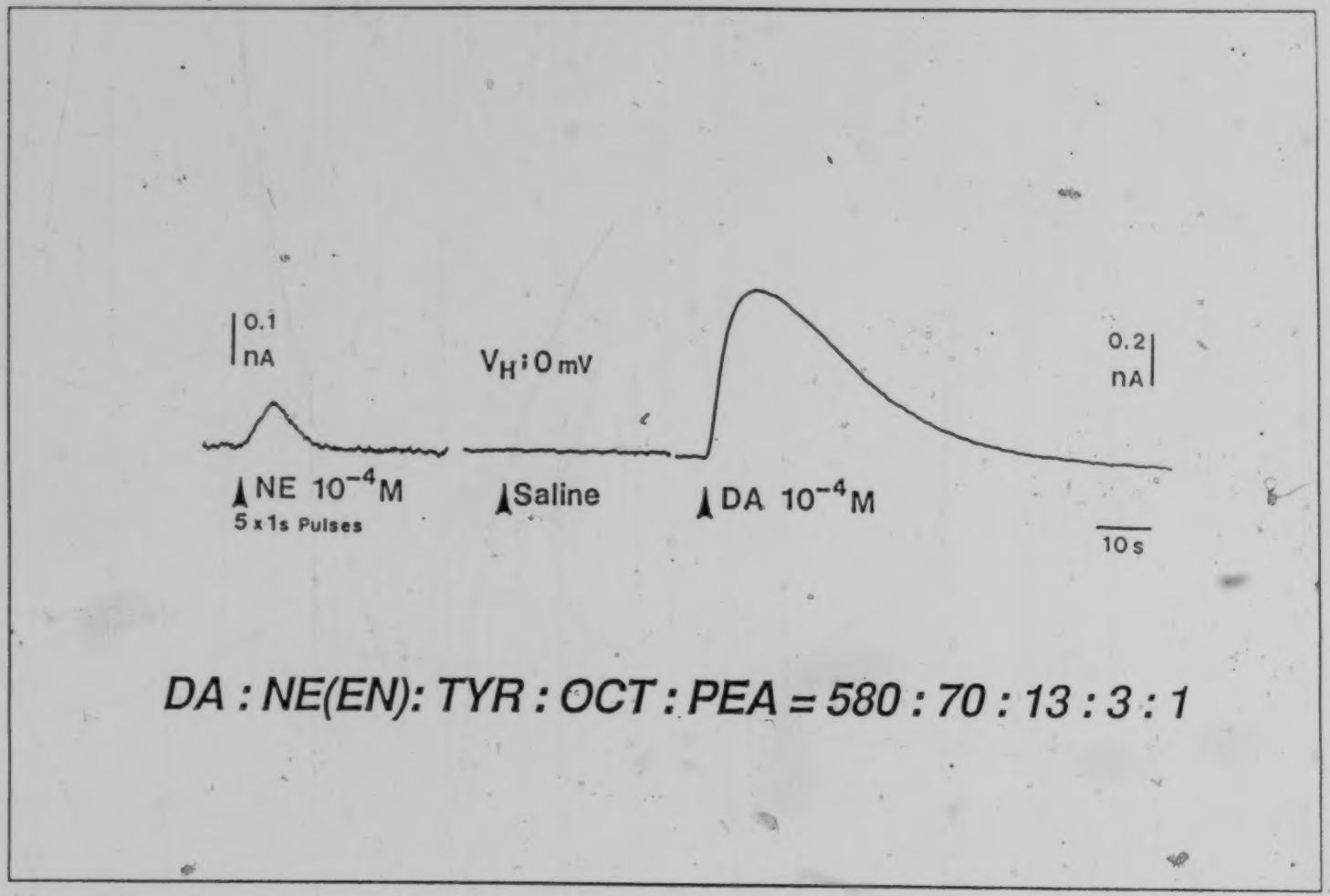

1.0
0.5
0.0
Dopamine response (nA)
- 7
- 5
- 3
Dopamine concentration (Log M)

Figure IV-4. Potency of various amines.

100 μM of norepinephrne (NE) applied for 5s to a SMN using a Picospritzer at 138 KPa produced 80 pA of outward current at 0 mV of membrane holding potential. The pressure pipette was then replaced with another that delivered saline to the same cell. The applied saline did not produce any significant response while 100 μM dopamine (DA) generated 640 pA of outward current. The potency ratio of dopamine to norepinephrine was calculated as 8. This method was employed for determining the potency order of several amines. The apparent potency order of amines is; dopamine, norepinephrine(epinephrine), tyramine (TYR), octopamine (OCT), and β-phenylethylamine (PEA). The potency ratio of two or three amines were determined from each cell and each ratio value was averaged from at least 3 different cells.



DA : NE(EN): TYR : OCT : PEA = 580 : 70 : 13 : 3 : 1

Table IV-1. Percentage of neurons giving an agonist current when various amines were applied.

AGONIST RESPONSE

	Number of cells Responding			Number of cells not Responding			Percent (%)
	10^{-4}	10^{-5}	10^{-6}	10^{-4}	10^{-5}	10^{-6}	Responding
DA (N=14)	9	3	2	0	0	0	100
NE (N= 3)	2	-	-	1	-	-	66
EN (N= 3)	2	-	-	1	-	-	66
TYR (N=12)	7	0	0	1	3	1	58
OCT (N=12)	4	1	1	5	0	1	50
PEA (N= 6)	2	-	1	3	-	0	50
DHPG (N= 3)	0	0	-	1	2	-	0
5HT (N= 3)	0	0	-	2	1	-	0

-, Not tested.

* Cells were judged to respond if they gave a detectable outward current (< 5pA) due to the applied agonists at either –20 or 0 mV of holding potential in VC mode.

DA, *dopamine*; NE, *norepinephrine*; EN, *epinephrine*; TYR, *tyramine*; OCT, *octopamine*; PEA, *phenylethylamine*; DHPG, *dihydroxyphenylglycol*; 5HT, *serotonin*.

ratios (Table IV-1). This table also shows that neither serotonin nor DHPG elicited responses from SMNs.

B. Specificity of dopamine's action.

The above results strongly suggest that these jellyfish neurons have an aminergic receptor and further that the receptor is fairly selective for dopamine. However, it is not sufficient to determine the selectivity of the receptor for dopamine from agonist experiments alone since there can be differences in the intrinsic activity of each ligand. Thus, different analogues may vary in their capacity to initiate a biological response after occupying the receptor. Therefore, the effects of several antagonists were examined to determine the selectivity of the receptor to dopamine (summarised in Table IV-2).

When a saline containing $1x10^{-8}$ M fluphenazine, a general dopamine-receptor blocker in the mammalian nervous system (Heiss et al., 1976; Seeman, 1981), was perfused over a cell, a 2s pulse of 10^{-6} M dopamine generated an outward current of 56 pA (Fig. IV-5A). The amplitude of this current was 23% less than that of the control experiment when the cell was perfused with the saline which was NASW mixed with the drug vehicle (DMSO) in the proper ratio. The dopamine response recovered when fluphenazine was washed out with saline. The D_2-blockers, haloperidol and spiperone, also decreased the dopamine response (Fig. IV-5C & Table IV-2). Interestingly, SKF 83566, a specific D_1-antagonist, increased the dopamine-induced outward current by about 12% in three out of four recordings (Table IV-2). The effects of antagonists to adrenoceptors were also examined since both norepinephrine and epinephrine induced far smaller but similar outward currents (Fig. IV-4). Antagonists to α-adrenoceptors, such as prazosine (α_1 antagonist) and yohimbine (α_2 antagonist), did not alter the dopamine response (Fig. IV-5B & Table IV-2); while propranolol, an antagonist to β-adrenoceptors, decreased the dopamine response by 16% (in 6 out of 10 recordings).

The action of these drugs was further investigated by recording the total membrane current while perfusing continuously with $1x\ 10^{-6}$ M dopamine. When a pulse of $1x10^{-8}$ M fluphenazine was presented to the cell during the plateau phase of the outward current (see inset to figure IV-3), the dopamine-induced current was

Table IV-2. The percentage change of the dopamine-induced current by several antagonists.

	CONCENTRATION (M)			
	10^{-8}		10^{-6}	
Prazosine	?		0	(n=3)
Yohimbine	?		0	(n=5)
Propranolol	?		−16±3	(n=6/10)*
SKF 83566	?		+12±3	(n=3/4)**
Fluphenazine	−16±3	(n=3)	−33±7	(n=7)
Haloperidol	−25±5	(n=5)	−52±8	(n=3)
Spiperone	−11	(n=1)	−25	(n=1)

? Not tested.

* Propranolol *reduced* dopamine responses in 6 out of 10 recordings and did not affect dopamine responses in the remaining 4 recordings. The mean value was obtained from the 6 recordings where propranolol reduced dopamine responses. The same data treatment were also employed for SKF 83566.

** SKF 83566 *increased* dopamine responses by an average of 12% in 3 recordings and did not affect dopamine responses in 1 recording.

One μM dopamine was applied for 2s, using a Picospritzer (pressure, 138 KPa), to evoke outward currents from cells which were clamped at −20 mV. The values are expressed as mean ± SE (%).

Figure IV-5. Antagonists of the dopamine responses.

Swimming motor neurons were exposed consecutively, using a rapid exchange perfusion system, to the following external solutions: saline (control), drugs tested, and saline. dopamine was applied at 1 μM for 2s using a Picospritzer at 138 KPa. Ten nM of fluphenazine reduced the dopamine responses of a SMN by 16% (**A**). On the other hand, 1 μM of yohimbine did not change the dopamine responses from another SMN (**B**). **C** shows that haloperidol decreased the dopamine response by 30 & 56%. Effects of several pharmacological agents on the dopamine response were measured in this way. These data are summarized in Table IV-2.

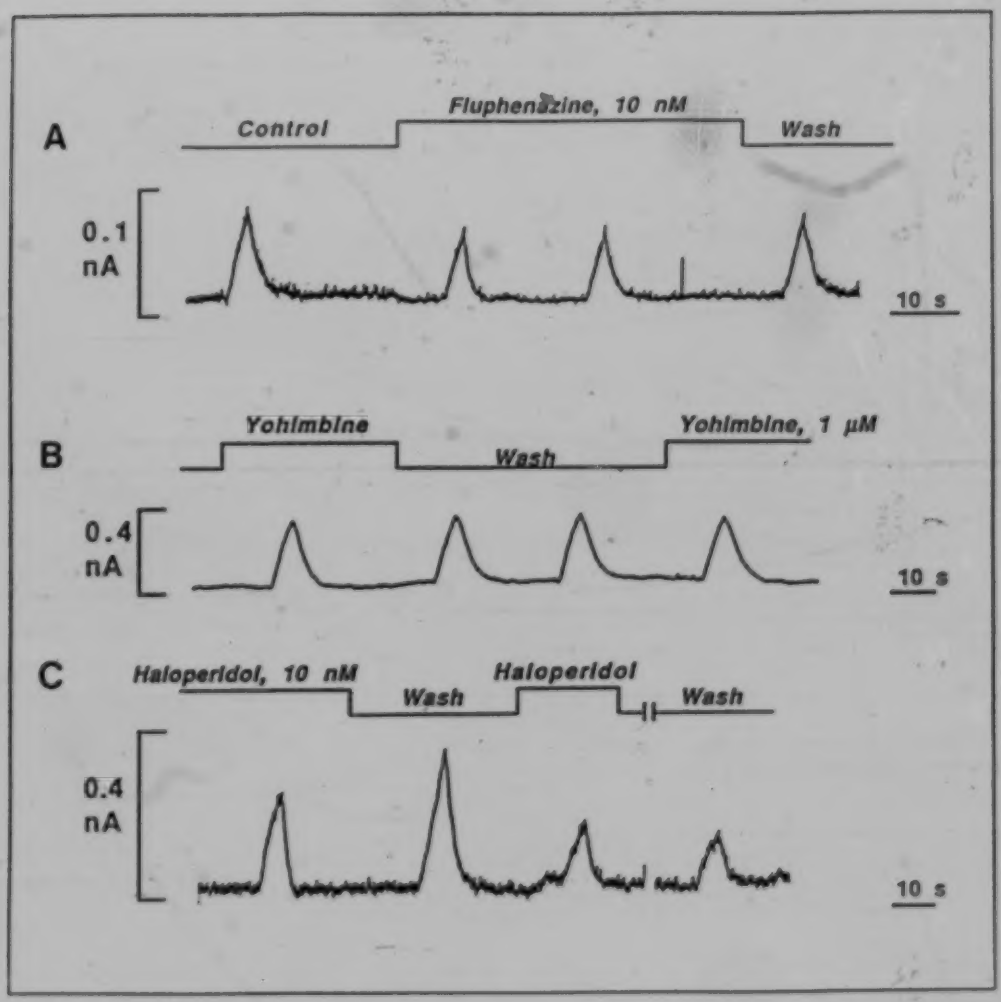
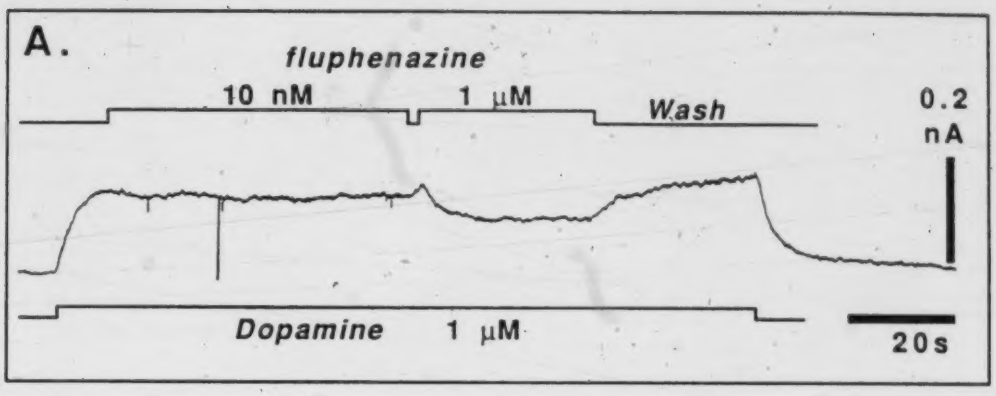

A
Control
Fluphenazine, 10 nM
Wash
0.1
nA
10 s
B
Yohimbine
Wash
Yohimbine, 1 μM
0.4
nA
10 s
C
Haloperidol, 10 nM
Wash
Haloperidol
Wash
0.4
nA
10 s

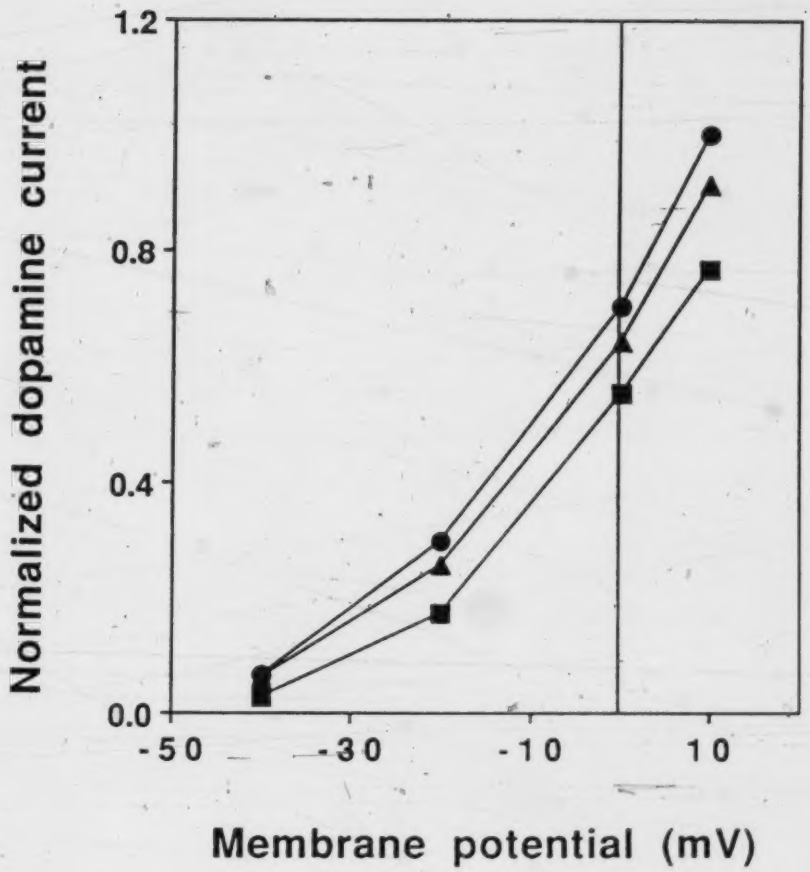
Figure IV-6. Fluphenazine as an antagonist of the dopamine-induced current.

A. Using a rapid solution exchange system, two different concentrations of fluphenazine were applied to a cell which was generating a prolonged dopamine-induced current at a holding potential of –20mV. The perfusion system consisted of 5 barrels (see materials and methods); the first and fifth barrel delivered 10 nM and 1 μM of fluphenazine, respectively, and the intermediate 3 barrels delivered saline. One μM of dopamine was applied continuously using a Picospritzer (pressure 138 KPa).

B. Current-voltage plot showing the effect of fluphenazine on the dopamine response of a swimming motor neuron. One μM dopamine was applied for 2s to the cell, using a Picospritzer (pressure, 138 KPa), to evoke dopamine-induced outward currents. Changing the solutions from normal artificial sea water (NASW, ●) to 10 nM fluphenazine (■) and returning to the NASW (▲) were accomplished using a rapid solution exchange system (see materials and methods). N=2.


A.
fluphenazine
10 nM
1 μM
Wash
0.2
nA
Dopamine
1 μM
20s

B.


Normalized dopamine current
1.2
0.8
0.4
0.0
-50
-30
-10
10
Membrane potential (mV)

decreased by 11% (Fig. IV-6A). After the cell was briefly exposed to NASW, a pulse of $1x10^{-6}$ M fluphenazine resulted in the current being reduced by 40%. As the fluphenazine was washed out, the amplitude of the outward current was completely restored (N=2, Fig. IV-6A). Figure IV-6B also shows the recovery of dopamine responses at different holding potentials after wash-out of fluphenazine.

The inset of figure IV-7 shows the effect of haloperidol on the dopamine-induced current at different holding potentials. Both haloperidol and fluphenazine reduced the dopamine-induced current in a concentration-dependent manner (Figs. IV-6A & IV-7; see also Table IV-2).

It was apparent that fluphenazine was more readily washed out than haloperidol. In most cases (n=7), cells did not recover their responsiveness to dopamine after they were exposed to haloperidol. In one case, a cell exposed to haloperidol generated a bigger response to dopamine when washed with saline (Fig. IV-5C). Even in this case, however, the cell did not recover its responsiveness to dopamine after the second application of haloperidol. In current-clamp recording mode, $1x10^{-8}$ M haloperidol reduced the dopamine-induced hyperpolarization by 20% (Figs. IV-8A & B). Removal of haloperidol only partially restored the excitability of the cell (Fig. IV-8C). This lack of reversibility was more noticeable when higher concentrations of haloperidol were used.

When applied at concentrations of $1x\ 10^{-5}$ M and greater all the neuroleptics that were applied, such as fluphenazine, haloperidol, domperidone, and spiperone, had a damaging effect. They often produced depolarization, an increase in spike frequency followed by decreased amplitude and longer duration spikes. Eventually there was a loss of the resting membrane potential and excitability. Even after prolonged washing, cells did not recover their activity (refer to Fig. IV-8C). Considering the high hydrophobicity of neuroleptics, it is probable that high concentrations of neuroleptics elicited a variety of nonspecific actions, such as fluidization of the membrane, which might destroy the membrane's excitability.

Figure IV-7. Current-voltage plot showing the effect of haloperidol on the dopamine-induced current.

The inset shows the effects of haloperidol on the dopamine-induced current occurring at two different holding potentials. [n.b. Different amplitude scales were used; 0.1 & 0.2 nA scales were for the outward currents at –20 mV & 20 mV, respectively.] One μM of dopamine was applied for 2s to the cell, using a Picospritzer (pressure, 138 KPa), to evoke dopamine-induced responses. Changing the solutions from NASW (●) to 10 nM (■) and 10 μM (▲) of haloperidol was accomplished using a rapid exchange system (See Materials & Methods). N=2.

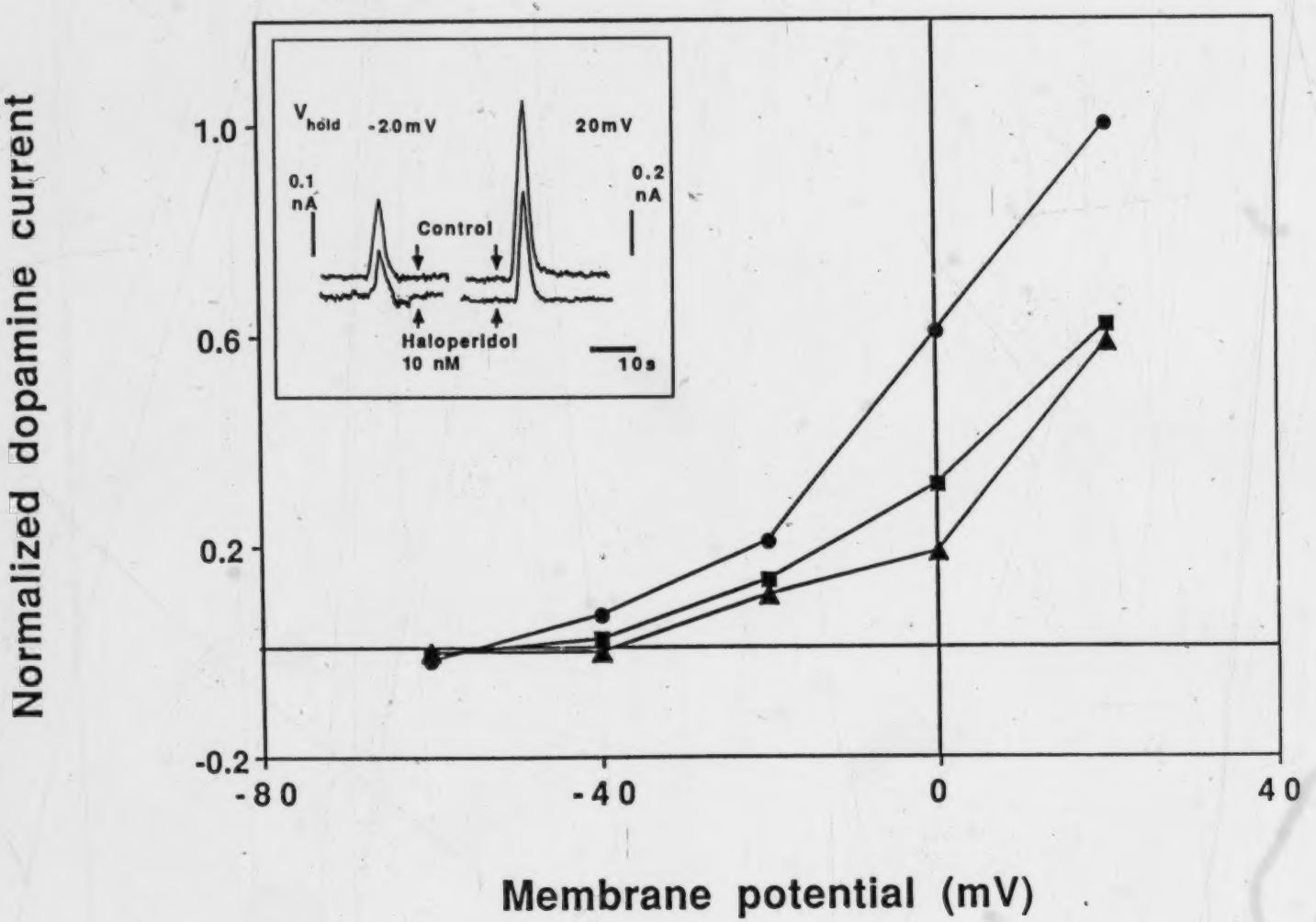
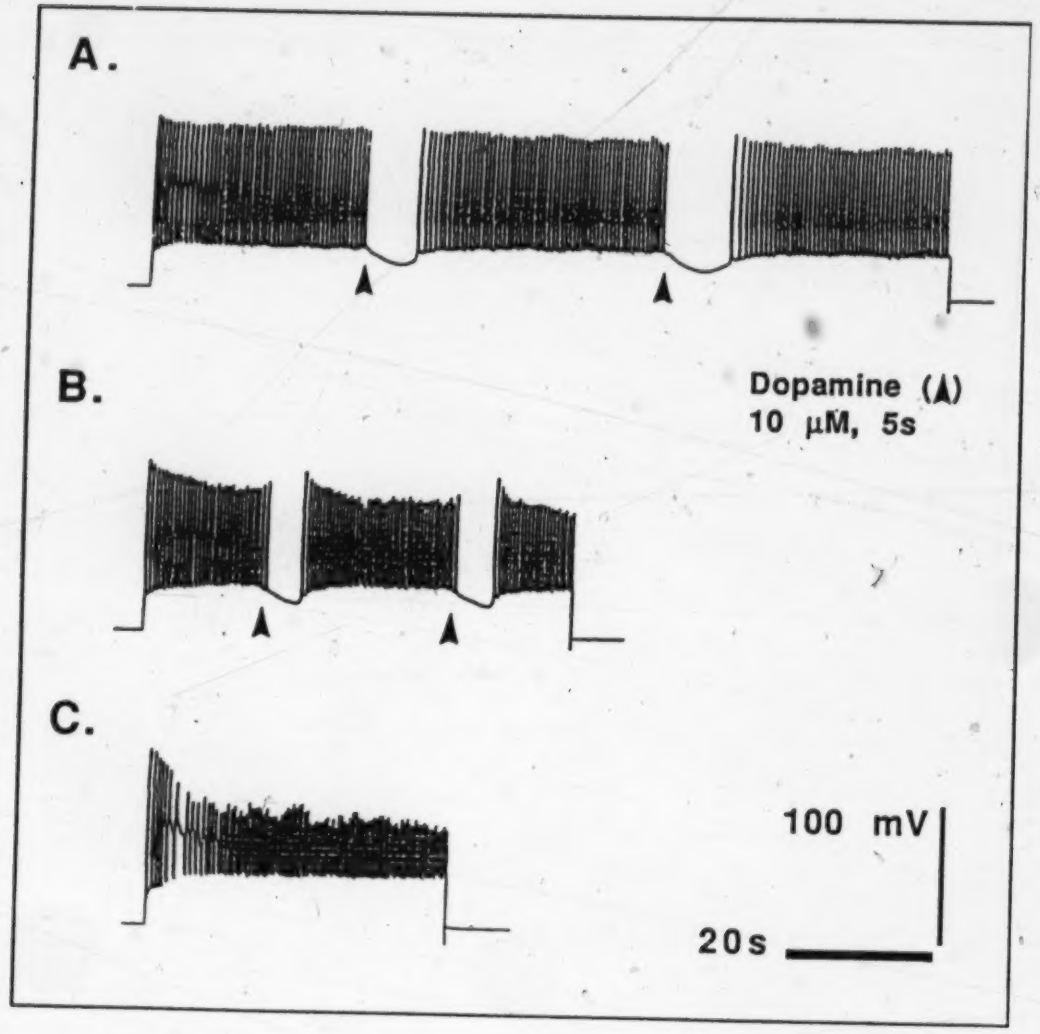

Normalized dopamine current
1.0
0.6
0.2
-0.2
-80
-40
0
40
Membrane potential (mV)
V hold -20mV
20mV
0.1 nA
0.2 nA
Control
Haloperidol 10 nM
10s

Figure IV-8. The effects of haloperidol on dopamine-induced inhibition.

A. The first application of dopamine (1 μM) applied for 5s using a Picospritzer (pressure, 138 KPa) resulted in membrane hyperpolarization (12.5 mV and 7.5s). The time (τ_p) to reach the peak amplitude was 5s. The second application of dopamine resulted in a membrane hyperpolarization of 12.5 mV and 10s. τ_p was 6s. During this experiment, the cell was continuously perfused with NASW by a rapid solution exchange system consisting of 5 barrels; the first to third barrels contained NASW and the fourth and fifth barrels contained 10 nM of haloperidol solution.

B. After the change of solutions from the NASW to haloperidol, the first application of dopamine generated a smaller and shorter membrane hyperpolarization of 10 mV and 5s. τ_p was 4.5s. The second application of dopamine also generated a smaller hyperpolarization of 10 mV and 6s; its τ_p was 5s.

C. Even 10 mins after changing the solutions from haloperidol to NASW, the cell did not recover its excitability completely on an anodal break stimulation which was obtained by switching from the voltage clamp mode (holding potential –60mV) to current clamp mode (see Chapter III). [n.b. during the washing step, the fourth and fifth barrels delivering haloperidol solutions were blocked using a three-way stopcock.]


A.
B.
Dopamine (▲)
10 μM, 5s
C.
100 mV
20s

DISCUSSION

Evidence is accumulating to suggest that dopamine acts as neurotransmitter or neuromodulator in the cnidarian *Polyorchis penicillatus*. First, the distribution of dopamine has been associated with the distribution of nerves in this jellyfish (see Chapter II). Second, dopamine applied to an identified cultured neuronal type from the CNS elicited membrane hyperpolarization and cessation or inhibiton of spiking through activation of K^+ channels (Chapter III). As a consequence of these studies it was necessary to establish whether the electrophysiological response induced by dopamine was mediated by an authentic receptor-dopamine interaction, or if it was mediated by a less specific action of dopamine on the membrane.

Receptor characteristics.

Several criteria for the identification of receptor sites have been proposed (Kahn, 1976; Kenakin, 1984; Laduron, 1984; Cooper et al., 1986). These mostly involve the properties of the receptor and can be roughly categorised into descriptions of the reversibility, saturability, and selectivity of the putative receptor.

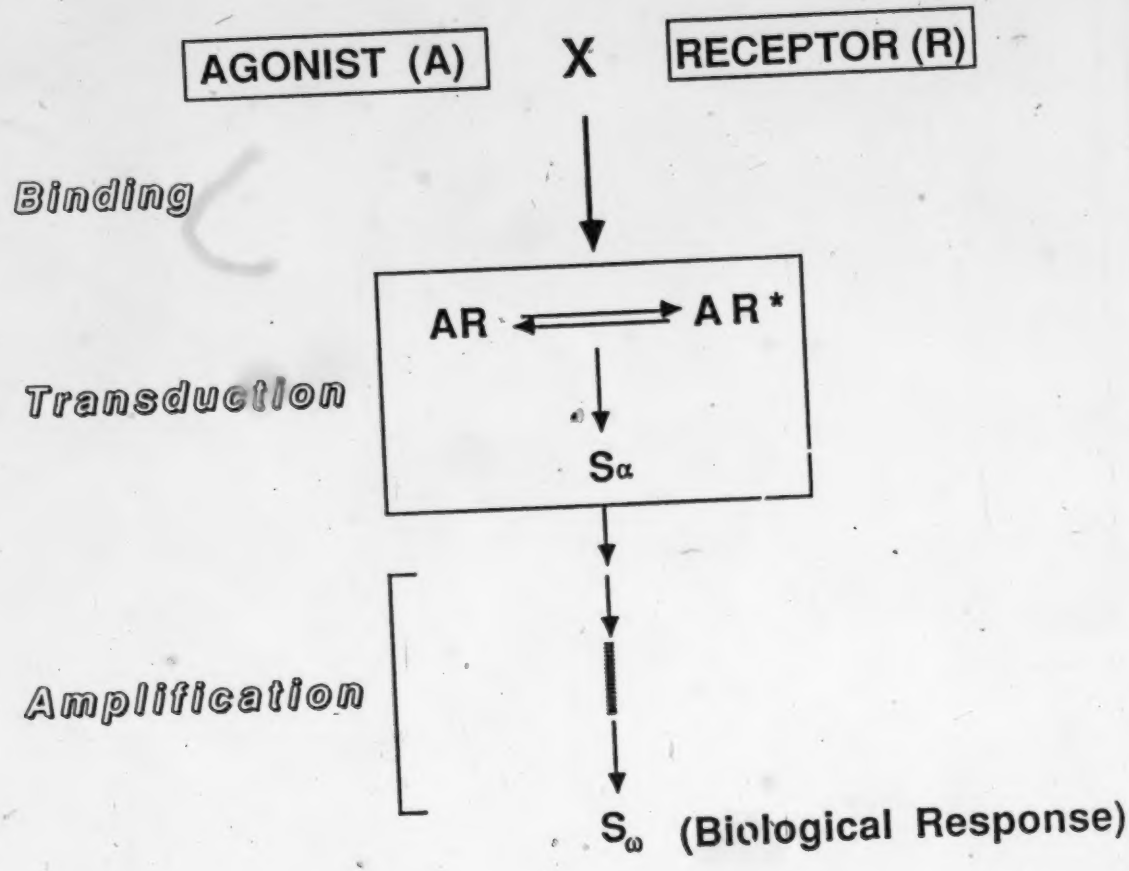
Transmitters, hormones and many drugs act in a reversible manner and the aminergic responses described in this study were, in most cases, no exception (Figs. IV-1 & 5). It was apparent that the dose-response relationship for the dopamine-induced outward current in SMNs was saturable, and additionally, the dose-response curve was sigmoidal. If one considered that the dose-response relationship seen here as being solely a result of receptor-ligand interactions, then the sigmoidality of the dose-response curve would indicate that the amine receptors are positively cooperative. The Hill coefficient (n) calculated from the Hill plot was 2.98, which was close to the apparent Hill coefficient (n_H) of 2.69. The apparent Hill coefficient was determined from the ratio (0.71) of the concentration of dopamine required for 90% of the maximal response to that required for 10% of the maximal response. Usually the next highest integer from the calculated Hill coefficient would represent the minimum number of binding sites. That is to say, the receptor behaved as if it possessed three binding sites with strong cooperativity. This interpretation, however, must be oversimplified since

the activation of receptors by agonists is not merely a binding process but can consist of multiple steps, such as binding (recognition), transduction, and amplification (Fig. IV-9). Thus the sigmoidal nature of the dose-response curve shown in Fig. IV-2B may represent the cooperativity of enzyme molecules involved in a second messenger cascade rather than that of receptor molecules. Also the dose-response relationship would not fit the Hill equation if an agonist activated several distinct channels. Yasui and his colleagues (1985) showed that a single Hill equation is not fitted by the dose-response relationship for GABA in frog sensory neurons where GABA activates three distinct chloride channels. Furthermore, the observed pharmacological response (the permeability change and some consequence of it) is more likely a function of the fraction of the total receptor in the 'active' conformational state than of the saturation of the receptor by dopamine. Accordingly, the saturability demonstrated using an electrophysiological method may not represent the finite number of the total receptors per cell but of 'active' receptors. My conclusion must therefore be limited to the following: that exogeneously applied dopamine produced its electrophysiological effects in a concentration-dependent manner, and that the receptor system showed saturability.

When motor neurons were exposed to long duration (greater than about 20s) pulses of dopamine (Fig. IV-3A) then the responses showed desensitization (Katz & Thesleff, 1957). This may be due to the conversion of an active agonist-receptor complex into an inactive complex (Katz & Thesleff, 1957; Feltz & Trautmann, 1982; Gero, 1983). Or dissociation of the agonist-receptor complex may be so slow that some receptors remain occupied and are not available for renewed agonist action (Paton, 1961). However, it should not be forgotten that drug action is a complex phenomenon: a drug-receptor interaction is only the first step and the last step is observable as the drug effect. Accordingly, desensitization may be occurring at steps beyond the agonist-receptor interaction. For example the process of desensitization at rodent β-adrenoceptors is known to include the rapid functional uncoupling of β-adrenoceptors from the stimulatory guanine nucleotide regulatory protein (G_s) and agonist-mediated internalization of receptors (Harden, 1983 : Sibley & Lefkowitz, 1987). Although not yet extensively studied, G-protein coupling (mainly inhibitory G proteins, G_i) has been described for several dopamine receptors (e.g Strange, 1990), thus it is possible that

Figure IV-9. A simplified diagram showing the steps that might be involved in receptor activation.

A, agonist; R, receptor; AR, agonist-receptor complex; AR*, active form of the complex; S, intermediate steps between AR* and final observable response.


AGONIST (A)
X
RECEPTOR (R)
Binding
AR
AR*
Transduction
Sα
Amplification
Sω (Biological Response)

both functional uncoupling of the receptor and receptor-internalization might occur in SMNs. Other possible sources of desensitization include the following: down-regulation of receptor number, an increase in the level of G_s protein (Jones & Bylund, 1988), and depolymerization of receptors. It is also possible that the diminution of the dopamine response does not reflect a receptor desensitization but just fatigue which is due to either the depletion of energy sources or the decrease of the potassium concentration gradient by excessive efflux of internal potassium following prolonged activation. The desensitization process may serve as a negative feedback control against prolonged receptor activation.

The bell-shaped concentration-response relationship (Fig. IV-3B) may be explained in two ways. First, it may imply that dopamine reacts with two receptor systems. If combination of dopamine with one receptor reduced the intrinsic activity (efficacy) of combination of dopamine with the other, then dopamine responses would become smaller as the concentration of dopamine becomes higher. This idea which has been described as 'noncompetitive auto-interaction' was proposed by Ariëns et al. (1957). Another possible explanation lies in 'rate theory' developed by Paton (1961). According to the theory, the rate of fade becomes faster than the response time as the concentration of a drug becomes higher. Therefore, the magnitude of the recorded peak response will begin to fall as dose increases further, being cut short by fade before the tissue has responded.

Selectivity of the receptor(s).

Frequently, judgments are made about the presence or absence of a receptor in a cell on the basis of the presence or absence of responses to a selective agonist. In this kind of experiment, the cell either responds or does not respond to the selective agonist. If the cell does not respond, it means either that the receptors for the particular selective agonist are not present in the cell or that the stimulus-response coupling of the cell produces insufficient amplification of the receptor stimulus to generate a response. If a selective agonist produces a response in a cell, then this constitutes circumstantial evidence that the receptor for which the agonist is selective is present in the cell. The relative potency of agonists has long been used (Barger & Dale, 1910) for receptor classification. However, it should be remembered that the apparent potency of agonists

can vary from cell to cell because of differences in receptor number and variations in the efficacy of the mechanisms coupling receptors to ionic channel proteins. On the other hand, the potency ratio for two "full" agonists is a powerful quantitative constant for drug receptor classification since it reflects only characteristics of the receptor and is tissue independent (Kenakin, 1984). In this study, several agonists were applied to the same neuron and their order of relative potency was determined in order to avoid errors due to cell to cell variability. The rank order of potency (Fig. IV-4 & Table IV-1) indicated that the receptor involved is selective for dopamine. However, if any of the amines are behaving as partial agonists then the potency order might change at agonist concentrations different from those tested .

The series of experiments using antagonists to block the dopamine-induced responses indicate that the receptors are selective for dopamine, though of course one can not be sure that there are not other dopamine-like substances that occur naturally and for which the receptors show an even greater selectivity. All of the antagonists used in this study have been used previously to classify mammalian receptor subtypes and thus they may not be equally useful as selective blockers of amine receptors in cnidarians.

The cellular neuropharmacology of dopaminergic receptors in invertebrates is poorly known and therefore it is not surprising that the inhibitory effect of dopamine was reduced in some recordings by propranolol, a β-adrenergic blocker in mammals. In the acinar cells of the cockroach (Ginsborg et al, 1976; House & Ginsborg, 1976), phentolamine, an α-adrenergic antagonist, reduces the dopamine response by about the same amount as does α-flupenthixol, a dopamine blocker. However, phentolamine did not block the effect of dopamine on the feeding response of the cnidarian *Hydra japonica* (Hanai & Kitajima, 1984). In this study by Hanai and Kitajima, neither flupenthixol nor chloropromazine, recognised as dopamine blockers in mammalian neurons, blocked dopamine's action but instead mimicked dopamine. In *Hydra* both ergometrine and DL-propranolol proved to be antagonists against dopamine receptors. Even though DL-propranolol blocked the action of dopamine, the authors suggested that dopamine's action is not mediated via a conventional β-adrenergic receptor since D-norepinephrine interacts with the receptor more strongly than its L-isomer. The more specific α_1 and α_2 adrenergic blockers, prazosine and yohimbine, had no effect on the dopamine-induced current in *Polyorchis*, suggesting that these receptors have features similar to

dopamine receptors in mammals.

The ergot alkaloids, such as ergometrine, were not used in this study since they appear to act as both agonists and antagonists at dopamine receptors in a variety of invertebrates (Hanai & Kitajima, 1984; Ascher, 1972; Buckett et al., 1990; Ku & Takeuchi, 1986; De Vlieger et al., 1986) and vertebrates (Pijnenburg et al., 1973; Woodruff et al., 1974).

The three neuroleptics used in this study (fluphenazine, haloperidol, spiperone) attenuated dopamine-induced responses, indicating that these responses were mediated by a dopamine receptor similar to mammalian D_2-receptors. This is somewhat surprising since there is evidence (Bokisch & Walker, 1986; Werkman et al., 1987; Audesirk, 1989) that the pharmacological profile of molluscan dopamine receptors differs from the D_1 and D_2 profile of mammalian receptors. It is therefore conceivable that the mammalian dopamine receptors have a more conserved structure than those in the molluscs.

The enhancing effect of SKF 83566 on the dopamine response may imply the presence of two subtypes of dopamine receptors in this jellyfish neuron. That is to say, it is possible that dopamine's effect, that is increased outward current, was enhanced by SKF 83566 binding to D_1-like receptors and decreasing an inward current. It is possible that under different preparation and recording conditions there would have been increased activation of D_1-like receptors, perhaps resulting in biphasic responses. For example, it is possible that D_1-like receptors are more susceptible to collagenase digestion than D_2-like receptors. It is also possible that, even though D_1-like receptors were occupied by dopamine, there was little or no coupling to an inward current because the nucleotides necessary for the adenylate cyclase cascade were washed out by dialysis from the recording electrode. The patch electrode used in this study did not include nucleotides.

Structure-Activity relationship

Assuming that the potency data obtained in this study reflect characteristics of amine-receptor interaction at the receptor level, then we can draw some conclusions about the structure-activity relationship of aminergic binding. First, since all

catecholamines and phenolamines produced hyperpolarizations in cultured SMNs but DHPG did not, it is likely that one of the functional groups of these agonists is the primary amine. At a pH of 7.4 (pH of normal saline used in this study), all the agonists have a positive charge on the amine group and therefore it can be expected that there are negatively charged amino acids such as glutamate and aspartate at the receptor site. Second, the relative importance of the hydroxyl group, located at ring position 3, is implied from the observation that dopamine and norepinephrine were more effective than tyramine and octopamine. Third, the hydroxyl group at ring position 4 also appears to aid binding, since tyramine generated larger effects than phenylethylamine. Fourth, norepinephrine is less effective than dopamine and octopamine is less effective than tyramine respectively, indicating that the β-hydroxyl group is likely to act in binding as a steric hindrance factor. Since serotonin did not evoke a response, there may be also a steric obstacle which does not allow the indole ring to fit in the active site. Finally hydrophobic interactions between the benzene moiety of catecholamines and some amino acids, such as tyrosine or phenylalanine, which may be located at the receptor site can be expected to play an important role in binding; this is confirmed by the lipophilic structure of the antagonists used.

In conclusion, dopamine generated its characteristic inhibitory action through a dopamine-selective receptor which was pharmacologically similar to the D_2 receptor described in mammalian cells.

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CHAPTER V.
GENERAL DISCUSSION

I have been told by a trustworthy friend that Maurice Ravel-the famous French composer .... asked his colleague Georges Auric to write with him a treatise of orchestration illustrated by a lot of examples, showing what one has to avoid instead of the usual good models. Indeed, error is often more instructive than success when you understand its genesis and can put your finger on the wrong step.

Bacq ZM (1980) TIPS 1: 141-3.

There has been extensive effort by neurobiologists for the past four decade to determine the identity of neurotransmitters in the cnidarian nervous system. However, we have to admit that no complete picture has emerged. I have used a variety of techniques to answer this apparently simple question. This multi-disciplinary approach has enabled me to present the most complete but not definitive evidence in support of DA as a cnidarian neurotransmitter. In this chapter I will integrate the major findings.

According to the HPLC results (Chapter II), dopamine is most abundant in nerve-rich tissues of *P. penicillatus* and is endogenous to the jellyfish. Also, the dopamine content in the tissue is likely dependent on the concentration of calcium ions in the bathing medium, suggesting that release of dopamine from dopaminergic cells is calcium-dependent. Calcium-dependent release of a transmitter appears to be common in many phyla (Llinás, 1980; Zucker & Haydon, 1988), although there is some controversy (see Hochner et al., 1989). The idea that dopamine is involved in neural transmission in *P. penicillatus* is further supported by the observation (Chapter III) that dopamine had specific electrophysiological effects on SMNs. Dopamine had two inhibitory effects on SMNs: first it caused hyperpolarization which was sufficiently strong to reduce or inhibit spontaneous firing; second it could reduce the effectiveness of excitatory synaptic inputs by reducing the input resistance of a SMN and hence shunt synaptic current. Dopamine's actions are quite specific. Dopamine affected only SMNs and was far more potent in exerting an inhibitory action than was any other amine. In addition, D_2-like receptors were pharmacologically identified to be responsible for dopamine's action. All these results strongly suggest that dopamine may play an important role in neural transmission of the jellyfish either as a neurotransmitter or neuromodulator. However, the biochemical results, showing the presence of dopamine in the jellyfish, do not necessarily mean that dopamine is localized in neurons.

To localize dopaminergic cells, several histochemical techniques (see Appendix 7) were employed. The results were ambiguous and no clear demonstration of monoamines in the inner neve-ring was possible. Tyrosine hydroxylase, one of the enzymes involved in catecholamine synthesis in mammals (Schwartz, 1985) and some invertebrates such as arthropod, mollusc and annelid (Leake & Walker, 1980), could not be demonstrated

by immunohistochemical methods. One possible explanation for the failure of these histochemical methods is that the autofluorescence that is often present in tissues of marine animals makes it difficult to distinguish the amine-induced fluorescence. In an attempt to circumvent this problem, I used a glyoxylic acid (GA) method with cultured cells which were pre-incubated in media containing precursors, such as tyrosine or DOPA. When the cultured cells were pre-incubated with DOPA, specific fluorescence was seen in a population of small cells (Appendix 8) which could not be identified. They might have been interstitial cells, epithelial cells, or small neurons. This result indicates that some cells, whether neurons or non-neuronal cells, have the ability to take up DOPA. In addition, these cells may have an aromatic amino acid decarboxylase which converts DOPA into dopamine. However, it should be noted that DOPA as well as dopamine can produce a fluorophore as a product of the reaction with glyoxylic acid. Recently, Mr. T. Diefenbach (pers. comm.) showed that some of the smallest cells with diameters less than 10 μm were excitable. It is possible that these excitable cells contain dopamine. From the histochemical results, it is evident that both SMNs and 'B' neurons do not contain dopamine. Therefore, dopamine is probably not involved in neural transmission either at the neuromuscular junction between SMNs and swimming muscle cells or at the neuroneuronal synapse between 'B' neurons and SMNs.

Intracellular recordings have identified two sources of inhibitory inputs to SMNs (Spencer, 1981; Arkett & Spencer, 1986). The similarity of dopamine-induced hyperpolarizations of SMNs to one of these two typos of IPSPs has been discussed in detail in Chapter III. In summary, the IPSP associated with the epithelial action potentials (accompanying the phenomenon of crumpling) is similar to the hyperpolarization induced by dopamine, being a slow, sustained membrane hyperpolarization followed by a spike with an afterhyperpolarization (AHP) greater than the AHPs of the previous spikes. These IPSPs were hypothesized to be electrically mediated since Mg^{2+} ions could not block them (Spencer, 1981). In Chapter III, I pointed out that a higher concentration (0.17 M instead of 0.08 M) of Mg^{2+} ions might have reduced or inhibited the IPSPs associated with crumpling. If so, then, the IPSPs associated with crumpling could be chemically mediated.

Crumpling, a contraction and shortening of the bell and an involution of the margin, usually initiated by mechanical stimulation of any part of the ectoderm, was observed

when nerve-rich tissues were dissected out in NASW. On the other hand, crumpling was rarely observed when tissues were dissected out in either Ca^{2+}-free saline or salines containing Ni^{2+} or Mg^{2+} ions. It is important to recall the HPLC results in Chapter II: the dopamine content in tissues was greater in samples pooled in either Ca^{2+}-free saline or salines including Ni^{2+} ions than in those in NASW. This indicates that the residual content of dopamine was lower in tissues when crumpling occurred during the dissection procedures.

A considerable body of information is available supporting the occurrence of an RFamide (Arg-Phe-amide carboxy-terminating peptides) family of peptides in distinct subpopulations of neurons in cnidarians (Spencer, 1989). In *P. penicillatus*, the RFamide immunoreactive neurons are found in all areas where there is smooth muscle and in the outer nerve-ring (Grimmelikhuijzen & Spencer, 1984). The RFamide immunoreactive neurons found in the outer nerve-ring penetrate into the inner nerve-ring where they approach the SMNs. However, the immunoreactive nerve-net is found to be distinct from other physiologically identified nerve-nets such as the 'B' and 'O' systems which are presynaptic to SMNs (Spencer, 1988). Spencer (1988) showed that 1 mM of neuropeptide applied to the SMN network elicited depolarization as well as a train of spikes. However, contradictory results were observed when the neuropeptides were applied to isolated SMNs in culture. For example, 1 μM poly-A peptide, "*Polyorchis* A peptide" which is one of the three RFamide peptides found in nerve-rich tissues of *P. penicillatus* (Grimmelikhuijzen et al., 1988), generated an outward current of 80 pA at V_H= –20 mV (Appendix 9) from a cultured SMN. One possible explanation is that the excitatory effect of RFamides *in vivo* may be produced by removal of a tonic inhibitory influence from a system presynaptic to the SMNs. Since the time courses of peptide-induced responses are different from those of dopamine-induced responses, SMNs are likely to have specific receptors for peptides. Anctil (1989) suggests that the evolutionary pathways for monoamines and peptides in cnidarian nervous systems may have evolved independently, since the distributions of monoamines and of the neuropeptide anthro-RFamide in *Renilla* do not overlap, and the two different putative transmitters target different tissues. Contrary to this, swimming motor neurons of *Polyorchis* may be possibly targeted by two different transmitters.

Preliminary experiments suggest that peptide-induced responses might also be K-dependent. An obvious way in which agonists at distinct receptors might open the same K-channel is by activating the same second messenger molecule. It is thus 'hypothesized' that dopamine and peptides bind to two distinct receptors present in SMNs of *Polyorchis*, share second messenger molecules and eventually open the same ionic channels. Such diversity and heterogeneity of receptors can be considered as evolutionary advantages for SMNs since they should endow the neurons with a greater integrative capacity.

What can be said at all can be said clearly,
and what we can not talk about we must consign to silence.

Ludwig Wittgenstein
In "the preface to the Tractatus Logico-Philosophicus".

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APPENDICES

Appendix 1. Electrochemical Detection.

The electrochemical detector is one of the most sensitive and the most specific LC detectors available. It measures either the conductance of the eluent or the current associated with the oxidation or reduction of solutes. To be capable of detection, the solutes must be ionic and they must be relatively easily oxidized or reduced.

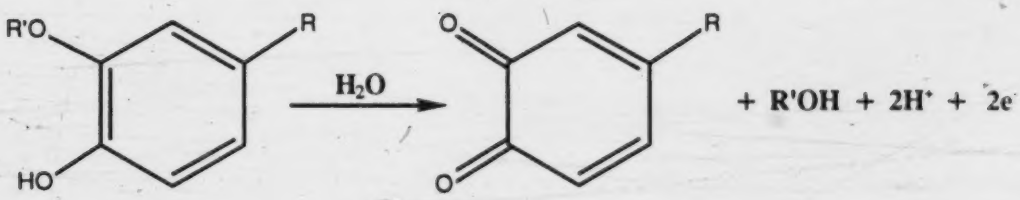
Types of Detectors. Three types of electrochemical detectors have been employed. *Conductivity detectors* measure the conductance (1/resistance) of the eluent rather than the conductivity. Conductivity can be used for the detection of inorganic or organic ions, usually after separation by ion exchange chromatography. The major problem with this is that ion exchange stationary phases need an ionic mobile phase which itself has quite a high conductance. Electrochemical detectors that measure current associated with the oxidation or reduction of solutes are called *amperometric* or *coulometric* detectors. The output from the detectors results from the electron flow caused by an electrochemical reaction that takes place at the surface of an electrode. The reaction can be either oxidation or reduction. The term 'EC detector' normally refers to these types rather than conductivity detectors. In *coulometric* detection, the electrochemical reaction proceeds to completion, exhausting all the reactant. In this state the current flow becomes zero and the total charge passed will be proportional to the total mass of material that has been reacted. In other words, coulometric detectors attempt to operate at essentially 100% efficiency, that is, to electrochemically oxidize or reduce 100% of the solute as it passes through the detector cell. In *amperometric* detection, the electrolytes flow past the electrodes. The solute, which constitutes the reactant, will be continously replaced throughout the elution of a peak. Thus, a current will be maintained while there is solute present between the electrodes. That is to say, amperometric detectors monitor flowrate-dependent oxidation or reduction current while attempting to convert as little as possible (<1%) of the solute in the process. These cells are of simple design, have a fairly small cell dead-volume, are flow- and temperature-dependent, and are essentially nondestructive, allowing other manipulations to be accomplished on the solute after separation (Mefford, 1985).

Types of Compounds The primary requirement for the use of EC detectors in HPLC is the type of compounds to be measured. That is, the compound of interest must be electrochemically oxidised or reduced (electroactive) within the potential range of the electrode material and solvent used. This requirement of EC detectors increases the selectivity of the technique. Some examples of compounds for EC detection are shown in the box.

Box 1.	Compound Type	Examples
	Phenols, amines	Neurotansmitters (Catecholamines, amino acids)
	Heterocyclic nitrogen compounds	cocaine, morphine, alkaloids, phenothiazines, purines
	Sulphur compounds	penicillins, thioureas
	Unsaturated alcohols	Vitamin C (ascorbate)
	Anions	I^-, $S_2O_3^{2-}$, SCN^-

Electrochemical Reaction. When current is passed through a solution, reactions occur at each electrode in which electron exchange takes place between the electrode and substances in the solution. At the cathode (-), substances in solution gain electrons (reduction) and at the anode (+) they lose electrons (oxidation). We can think of the cathode and anode as a reducing agent and an oxidizing agent respectively whose strength depends on the value of the electrode potential. A cathode becomes a stronger reducing agent as its electrode potential becomes more negative and an anode becomes a stronger oxidizing agent as its electrode potential becomes more positive. Oxidation and reduction result in different directions of current flow: for reduction, electrons flow out of the electrode, the cathode (a reducing current or cathodic current) and for oxidation, electrons flow into the electrode, the anode (an oxidising current or anodic current). In practice, it is difficult to use EC reduction as a method of detection in HPLC. Oxygen is very easily reduced, and if it is present in the mobile phase it will create a background current thousands of times larger than the current due to the solutes. To prevent this, oxygen would have to be carefully removed. This can be done, but it is not easy in practice. Most of the EC applications are, therefore, oxidations. Another important consideration with EC detectors is that the mobile phase used must have fairly high conductance.

Catecholamines are known to electrochemically oxidize to the corresponding orthoquinones at the surface of the electrode, according to the following reaction (Krstulovic, 1982):

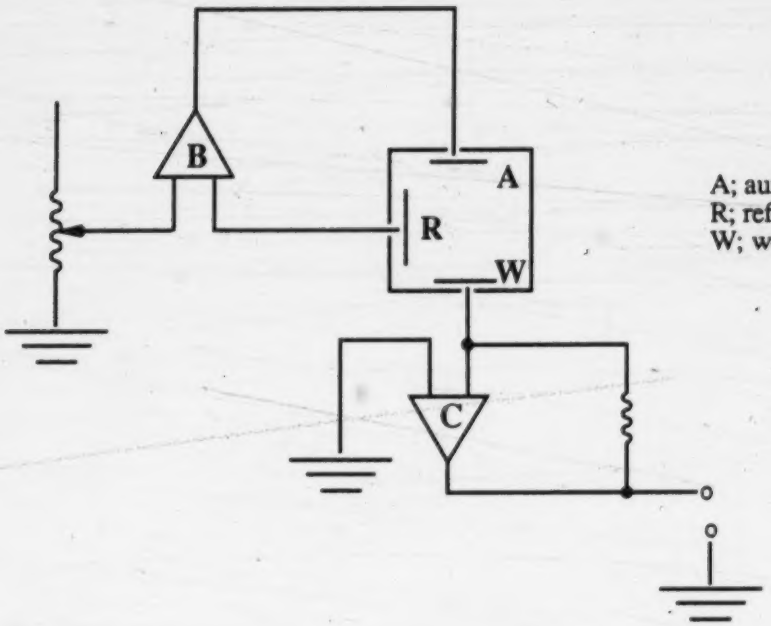


The instantaneous anodic current is directly proportional to the number of solute molecules in contact with the interface per unit time.

The reaction at the surface of the electrode is extremely rapid and proceeds almost to completion. This results in the layer close to the electrode being virtually depleted of reactant. Consequently a concentration gradient is established between the electrode surface and the bulk of the solution. This concentration gradient results in the solute diffusing into the depleted zone at a rate determined by the concentration in the bulk solution. As the current generated at the electrode surface depends on the rate at which solute reaches the electrode, the detector exhibits a linear response with respect to solute concentration.

Basic Electrochemical Detector Electronics. The basic circuit for an electrochemical detector is shown in Box 2. The potentiostatcircuitry for EC detection is illustrated more in detail by Mefford (1985). The auxiliary electrode A is held at a fixed potential by amplifier B the voltage being selected by potentiometer P. The potential near the auxiliary electrode is sensed by the reference electrode R. The reference electrode allows the voltage to be compensated for changes in the mobile phase conductivity. On arrival of a solute at the surface of the working electrode that can be either oxidized or reduced, a current is developed which is converted by amplifier C to a voltage output, V. This output can either be fed directly to a suitable recorder or through an appropriate attenuator system, a filter circuit and then to the recorder. The EC detector is extremely sensitive , but suffers from two main drawbacks. First, the mobile phase has to be extremely pure, in particular, free of oxygen and metal ions. Second, by-products of the oxidation or reduction processes are often absorbed onto the surface of the electrodes and thus, if quantitative activity is required, frequent calibration is necessary. Ultimately the electrodes have to be cleaned, usually by mechanical abrasion, and replaced in the cell.

Box 2. Basic Circuit for an Electrochemical Detector.



A; auxiliary electrode
R; reference electrode
W; working electrode

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Appendix 2. GCMS

To obtain a mass spectrum, the sample must be vaporized, ionized, and then (provided the substance is molecular) allowed to fragment or decompose. The various ions must then be separated according to their mass-to-charge ratios (m/z-values) and finally detected. The instrumentation necessary to accomplish these requirements has four major components: (a) inlet systems for vaporization, (b) a source that serves to ionize and then detain the ions for a short period of time (usually about 1 μsec) so that fragmentation may occur, (c) a method of mass analysis, and (d) a detection system. A brief review of the four components of the GCMS system used in my research is given in this appendix.

1. The Inlet System.

There are three ways to introduce samples to analytical mass spectrometers. 1. Using a batch inlet for gases and liquids and for solids of moderately high vapor pressure. 2. Using a direct inlet for high-molecular-weight nonvolatile solids and for thermally unstable compounds. 3. Using a gas chromatograph (GC).

Components separated by a GC using packed columns can be admitted to the source of a mass spectrometer after enriching the eluent vapor, i.e., separating the helium carrier gas from the sample vapor with a molecular separator. The most common design is is a jet separator made entirely of glass to prevent adsorption or decomposition of the sample molecules. As the helium and sample pass through the constriction (jet), located below the vacuum-pump outlet, they expand into the low pressure, glass envelope. Because helium diffuses much more rapidly than larger organic molecules, it is preferentially pumped away. The trajectory of the sample molecules is less affected, and most continue directly into the tube leading to the mass spectrometer source. The entire jet assembly is contained in an oven, and the temperature is controlled to be slightly greater than that of the GC column.

Capillary columns involve much slower flow rates than packed columns (1 to 2 ml/min compared to 30 to 50 ml/min). As a result, an interface is often not required. In fact the new, fused-silica capillary columns can be threaded directly from the GC oven into the source ionizing chamber, permitting 100% efficiency. A fast scanning mass analyzer is necessary, because the width of capillary GC peaks is of the order of a few seconds.

2. Ionization.

The ion source (the region where the sample ionizes and fragments) is the heart of the mass spectrometer. There are several methods to vaporize, ionize, and fragment the sample.

A. Electron-Impact Ionization. The most common method of ionization is by electron impact; that is, a high-energy electron beam dislodges an electron from a sample molecule to produce a positive ion:

$$M + e^- \longrightarrow M^+ + 2e^-$$

where **M** = molecule under study, M^+ = molecular or parent ion.
The beam produces M^+ in a variety of energy states. Some molecular ions are produced with rather large amounts of internal energy (rotational, vibrational, and electronic) dissipated by fragmentation reactions; for instance,

$$M^+ \longrightarrow M_1^+ \longrightarrow M_3^+$$
$$\longrightarrow M_2^+ \longrightarrow M_4^+$$

where M_1^+, M_2^+ ,... are lower-mass ions. Other molecular ions resist decomposition because they are formed with insufficient energy for fragmentation. It should be noted that most fragmentation processes are endothermic, and thus low-energy molecular ions will not fragment in the source and will be detected as the molecular mass.

An electron gun accelerates and focuses electrons emitted by a thin, red-hot filament usually made of rhenium or tungsten. The electrons are accelerated by placing a negative bias of 70 V on the filament, producing a beam with a gaussian distribution of kinetic energies around a maximum at 70 eV (1 eV = 23.06 Kcal/mole). Because most covalent molecules have ionization potentials of around 10 eV, 70 eV electrons are sufficient to dislodge an electron from one of the high-energy molecular orbitals and produce a molecular ion with a distribution of internal energies. Molecular ions in high-energy states can then decompose, producing various fragment ions.

B. Desorption Ionization. The requirement that the sample be converted to gas-phase ions can be the chief drawback for thermally sensitive solids (e.g., carbohydrates, peptides, nucleic acids, organometallics). New methods of ionization in which the sample ions desorb directly from the solid state to the gaseous state without decomposition have been developed recently.

a. Thermal desorption mass spectrometry. The sample is placed on a thin wire and heated very rapidly (hundreds of degrees per second). At high temperature, desorption or evaporation is kinetically more favorable than decomposition. This method can be used to convert organic salts to the gas phase.

In **field desorption mass spectrometry,** high electric fields are used to facilitate desorption. Heating of the wire, coupled with application of the high field,

causes solid-state ions to desorb directly into the gas phase.

b. Particle bombardment method. Lower energy particle bombardment has taken two forms. The first, **fast-atom bombardment (FAB),** uses a discharged beam of argon or xenon atoms travelling at 6000 eV to bombard the sample, which is dissolved in a nonvolatile solvent, such as glycerol and placed on the metal tip of a probe admitted to the back of the ion source. The second form of the technique is called **organic** or **molecular secondary-ion mass spectrometry (SIMS).** Here, fast ions, such as argon, xenon, or cesium cations are used to bombard the sample, which is either coated on a silver grid or dissolved in glycerol as for FAB.

C. Field Ionization Mass Spectrometry (FIMS). A small wire or sharp-edged anode is mounted at the input end of the ion gun. Very large electric field (10^5 V/cm) is applied between the anode and cathode. The electric field is then sufficient to remove an electron from gaseous molecules in the field. The high field distorts the potential-energy surfaces of the sample molecule so that an electron may be quantum mechanically tunneled through the energy barrier to the anode. The practical consequence is that the molecular ions, which are not excited, are formed and thus little fragmentation occurs. Most compounds give an abundant M^+ or $(M + 1)^+$, by field ionization, and considerably simplified fragmentation patterns.

D. Chemical Ionization. Instead of ionizing with an energetic electron beam, chemical ionization occurs via ion-molecule reactions. This method requires a reactant gas (Munson, 1971) which can produce a set of ions which are either 'nonreactive' or only very slighly reactive with the reaction gas itself, but which can react with other materials. Methane was employed as the reactant gas [originally named as a reaction gas (Munson & Field, 1966)] in this study. This method also requires a large amount of the reactant gas and a small amount of the sample; the ratio of reactant gas to sample should be of the order of 1000. Under these conditions, practically all of the high-energy electrons passing through the gas within the source will ionize methane, and ionization of the sample by electron impact will be negligible. The direct ionization of methane with high-energy electrons (E > 50 eV) gives several ions (CH_4^+, CH_3^+, CH_2^+, CH^+,...). However, CH_4^+ and CH_3^+ are by far the most abundant of these ions, being formed in approximately equal amounts and comprising about 90% of the total ionization. CH_4^+ and CH_3^+ react rapidly with methane to produce other ions, CH_5^+ and $C_2H_5^+$, respectively. Since CH_5^+ and $C_2H_5^+$ react slowly or not at all with methane and comprise 90% of the total ionization, it is the reactions of these ions with the sample molecules which produce the major part of the chemical ionization mass spectrum of the sample. CH_5^+ and $C_2H_5^+$ react mostly by proton- or hydride-transfer

reactions

$$CH_5^+ + BH \longrightarrow BH_2^+ + CH_4$$
$$C_2H_5^+ + BH \longrightarrow BH_2^+ + C_2H_4$$
$$C_2H_5^+ + BH \longrightarrow B^+ + C_2H_6$$

Since CH_4 is a weak Brönsted base and CH_5^+ is a strong Brönsted acid, proton transfer reactions to stronger bases than CH_4 will occur. These rapid proton and hydride transfer reactions are exothermic and the excess energy will be distributed between the ionic and neutral products. Energy remaining in BH_2^+ or B^+ is enough to decompose the ions (BH_2^+ or B^+) into set of ions. The set of ions produced, BH_2^+ and B^+ comprise the **positive ion chemical ionization (PCI)** mass spectrum of BH. Ethyl addition ions, $(M + 29)^+$, are observed for some compounds in methane PCI mass spectra.

Bombardment of methane at 1 Torr with 100 eV electrons generates CH_5^+ and $C_2H_5^+$ ions in high abundance as described above. These reactions can be described as follows:

$$2CH_4 + 2e \longrightarrow CH_4^+ + CH_3^+ + H. + 2e^* + 2e$$
$$CH_4^+ + CH_4 \longrightarrow CH_5^+ + CH_3$$
$$CH_3^+ + CH_4 \longrightarrow C_2H_5^+ + H_2^+$$

Formation of each positive reagent ion is accompanied by the production of a low energy electron. Each ionizing event removes about 30 eV from the bombarding electron and the energy of the incident electron beam is further reduced by additional non-ionizing collisions with neutral methane molecules. Thus, operation of a mass spectrometer under methane CI conditions should afford a mixture of both positive reagent ions and a population of electrons with near thermal energies. Formation of negative ions by interaction of electrons and sample molecules can occur by three different mechanisms, such as resonance electron capture, dissociative resonance capture and ion-pair production.

Hunt and Crow (1978) pointed out that formation of a negative ion by resonance electron capture is ca. 400 times faster than an ion molecule reaction. Accordingly, the negative sample ion current should exceed the positive ion sample current by a factor of ca. 400, if sample molecules are converted to stable positive and negative ions on every encounter with CH_5^+ and thermal electrons. Hunt and Crow (1978) reported that the sensitivity of negative ion chemical ionization (NICI) GCMS is 10 to 100 times higher than that of EI.

CI is potentially more sensitive than EI, for two reasons. First, the ionization of the

sample is concentrated in a few ions rather than dispersed over hundreds of ions as in the EI of complex molecules. Second, the efficiency of EI is approximately 0.1%; that is, only one out of every thousand sample molecules is ionized. The un-ionized sample molecules are lost to the vacuum pumps. Because CI takes place by ion-molecule reactions, ionization efficiency could be raised significantly by increasing the reactant gas pressure and thus the concentration of reagent ions, and by extending ion residence times. In principle, the efficiency of CI should be 1000 greater than that of EI.

3. Mass Analysis.

A number of methods of mass analysis can be used in mass spectrometry.

A. Mass spectrometer with magnetic sectors. The most common type involves a magnetic sector. Once outside the source, the ion beam moves down a straight, evacuated tube toward a curved region placed between the poles of a magnet. This region is called the magnetic sector and its purpose is to disperse the ions in curved trajectories that depend on the m/z of the ion. Low mass ions are deflected most, and the heavier mass ions the least. Mass spectrometers with only a sector magnetic field for mass analysis are known as **single-focusing mass spectrometers.** A well-designed single-focusing spectrometer may have a resolution as high as 5000. In mass spectrometry, resolution R is defined as

$$R = m/\Delta m$$

where Δm = mass difference between two resolved or separated peaks,

m = nominal mass at which the peaks occur.

A resolution of 5000 would indicate that m/z = 5000 would be resolved from m/z = 5001 (or m/z = 50.00 from m/z = 50.01).

If a resolution greater than 5000 is required, a **double-focusing mass spectrometer** is necessary. Two factors that limit resolution in the single-focusing instruments are the angular divergence and spread in kinetic energy of the ion beam as it leaves the ion gun. The various ions in the beam always have a small spread of kinetic energies because they are formed in different regions of the ionization chamber and, therefore, experience different total acceleration. To correct these aberrations, an electrostatic analyzer or sector is introduced. This device consists of two cylindrical electrodes; a positive voltage is applied to the outer one and a negative voltage of equal magnitude to the inner one. The radius of curvature of an ion beam through this sector is determined by the kinetic energy of the beam for a constant voltage; the higher the kinetic energy, the greater the radius. Ultimately, high energy ions (+) will be deflected so little that they will impinge on the positive electrode. Ions (+) of low kinetic energy are discharged on the negative electrode. Thus, the electrostatic analyzer serves as a kinetic-energy analyzer. The electrostatic analyzer serves to sort out ions

of equal kinetic energy and brings them to a common focus. Thus, a beam emanating from a single point (the source) is brought to focus at many points, each representing a common kinetic energy.

The mass spectrometer used in this study, VG 7070E, is a double-focusing instrument.

B. Time-of Flight (TOF) Mass Analysis. TOF spectrometers are equipped with a modified electron-impact source and a long, straight flight tube. Different masses are distinguished by their different arrival times at the detector located at the end of the tube.

The advantage of a TOF mass spectrometer is its rapidity in scanning a spectrum.

C. Quadrupole Mass Analyzers. An ion beam from a conventional source is injected into a dynamic arrangement of electromagnetic fields. Certain ions will take a "stable" path through the analyzer and be collected; others will describe "unstable" paths and be filtered out. **The quadrupole** is one example of this type of mass spectrometer (***path-stability mass spectrometer; mass filter***) and has become quite popular in recent years , especially in the aea of GCMS.

Quadrupoles have a number of distinctive advantages. First, the path does not depend on the kinetic energy or the angular divergence of the incoming ions; therefore, these instruments have high transmission. Second, they are relatively inexpensive and compact. Third, a complete scan can be achieved very rapidly since only a change in voltage is required.

4. Methods of Ion Detection.

The most useful and sensitive method of detection is to focus the mass -analyzed beam of ions on an **electron multiplier.** Another method is to place a photographic film on the focal plane but this is rarely used now.

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Appendix. 3. Significance of DHBA as an internal standard.

The internal standard technique is widely used in chromatography. In this approach, an accurate amount of a known compound is added to the sample solution prior to analysis. Thereby errors in the analytical measurement are often reduced, since any loss of sample is compensated by the loss of an equivalent amount of internal standard. Instead of the absolute value of the peak height, the ratio of the peak height of the compound to the peak height of the internal standard is used in calibration and in the evaluation of the unknown samples. However, rapidly developing HPLC technology questions the importance of internal standard method. For example, the modern sample loop injector in HPLC performs with excellent precision, limiting the variation of the injection volume, one major source of the methodological variations which have previously been overcome by the internal standard technique. Another source is the variation of the extraction. However, this is no guarantee that precision will be improved by using an internal standard, since the extraction behavior of the internal standard and the substance of interest can be different and make a high relative standard deviation, although both compounds are similar in their chemical structure.

Based on the law of propagation of error, Haefelfinger (1981) derived a formula which can determine the significance of an internal standard. Briefly, this states that the standard deviation of the peak height of a substance (α) should be larger than that of the quotient (Q) of the substance to an internal standard β ($Q=\alpha/\beta$), if the internal standard technique improves the precision of a method;

$$S_{Q,rel} < S_{\alpha,rel} \quad \text{or} \quad S^2_{Q,rel} < S^2_{\alpha,rel}$$

where $S_{\alpha,rel}$ is the relative standard deviation of α (the standard deviation of α devided by mean α) and $S_{Q,rel}$ is the relative standard deviation of the quotient Q. Using the theory of propagation of error, he finally reached the following formula;

$$S_{\beta,rel} < 2rS_{\alpha,rel}$$

where $S_{\beta,rel}$ is the relative standard deviation of β, an internal standard, and r is the correlation coefficient between α and β. Only if this relation holds true will an internal standard procedure improve the precision of a particular method. Otherwise, it would be better to avoid the use of an internal standard, since it is often easier and less time-consuming to look for a suitable external calibration approach.

DHBA was chosen as the internal standard in this study for the following reasons; it was completely resolved in the chromatogram from the other known and unknown

substances, it eluted near the peak of DA, it is chemically similar to DA, and it is chemically stable. The correlation coefficient (r) between DHBA and DA was about 1.0 (0.998). For the unextracted standard sample in Table II-3, $S_{DA,rel}$ is 0.01 (0.01/0.71) and $S_{DHBA,rel}$ is 0.02 (0.02/0.94). Therefore, $2rS_{DA,rel}$ is almost equal to $S_{DHBA,rel}$. On the other hand, for the extracted standard sample, $2rS_{DA,rel}$ (0.20) is much greater than $S_{DHBA,rel}$ (0.05). This result supports the use of DHBA as an internal standard to quantify DA from alumina-extracted samples.

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Appendix. 4. Tissue Levels of Dopamine in Mammalian Brains.

Tissue	DA level (µg/g wet.)	Method	Species	Reference
Striatum				
	4.53	HPLC-ED	rat	Oka et al. ('84)
	7.95	HPLC-ED	rat	Kontur et al. ('84)
	8.80	HPLC-ED	rat	Zaczek & Coyle ('82)
	9.50	GCECD	rat	Origitano & Collins ('84)
	665*	NICIMS	rat	Wood ('82)
Hypothalamus				
	0.10	HPLC-ED	rat	Oka et al. ('84)
	0.96	HPLC-ED	rat	Freed & Asmus ('79)
	0.29	GCECD	rat	Origitano & Collins ('84)
Whole brain				
	0.89	HPLC-ED	rat	Ishikawa & McGaugh ('82)
	0.97	HPLC-ED	rat	Sasa & Blank ('77)
	0.80	GCMS	rat	Weintraub et al. ('75)
	0.77	Fluor.	rat	Fleming et al. ('65)
	0.87	Fluor.	rat	Wiegand & Perry ('61)
	0.82	Fluor.	rat	Smith ('63)
	1.33	Fluor.	rat	Agrawal et al. ('68)

HPLC-ED, liquid chromatograph with electrochemical detector; Fluor., fluorimeter; GCECD, gas chromatograph with electron capture detector; NICI, negative ion chemical ionization.
** This was expressed in pmol/mg protein.*

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Appendix 5. Tissue Levels of Dopamine in Mammals.

Tissue	DA level (µg/g wet.)	Species
Adrenal medulla	0	man
	17	cattle
Carotid body	20-40	rabbit
Ganglia:		
superior cervical	0.82	cat
stellate	1.32	cat
coeliac	2.2	dog
renal	2.0	dog
Kidney:		
total	0.07	cat
total	0.03	dog
cortex	0.08	dog
medulla	0.03	dog
Jejunum:		
total	0.16	dog
mucosa	0.20	dog
muscularis	0.05	dog
Colon	0.30	dog
Lung	0.06	dog
	0.60	man
Mesenteric artery	0.03	dog
Heart:		
total	0.16	dog
sinoatrial node	2.06	dog
right atrium	0.11	dog
left ventricle	0.10	dog
Plasma (ng/ml)	0.98	man
	1.60	dog
Urine (ng/ml)	473	man
	104	dog

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Appendix 6. Definition of leak resistance in this study.

Figure A shows a simplified schematic diagram of the whole cell recording situation and figure B an equivalent electrical circuit. Without consideration of a cytoplasmic resistance, a total resistance of the circuit (R_T) would be:

$$R_T = R_e + [R_s \times \{R_p + R_x\}]/(R_s + R_p + R_x) \quad \text{------} \quad (1)$$

when $R_x = R_m \times R_L/(R_m + R_L)$; R_e, electrode resistance; R_s, seal resistance; R_p, patch membrane resistance; R_m, membrane resistance; R_L, leakage resistance.

The number of ionic channels over the membrane patch should be very small. The patch membrane resistance (R_p), thus, is expected to be extremely high with respect to the seal resistance (R_s) before rupture of the membrane patch. The total resistance (R_T) would then be simplified as the sum of the electrode resistance (R_e) and the seal resistance (R_s);

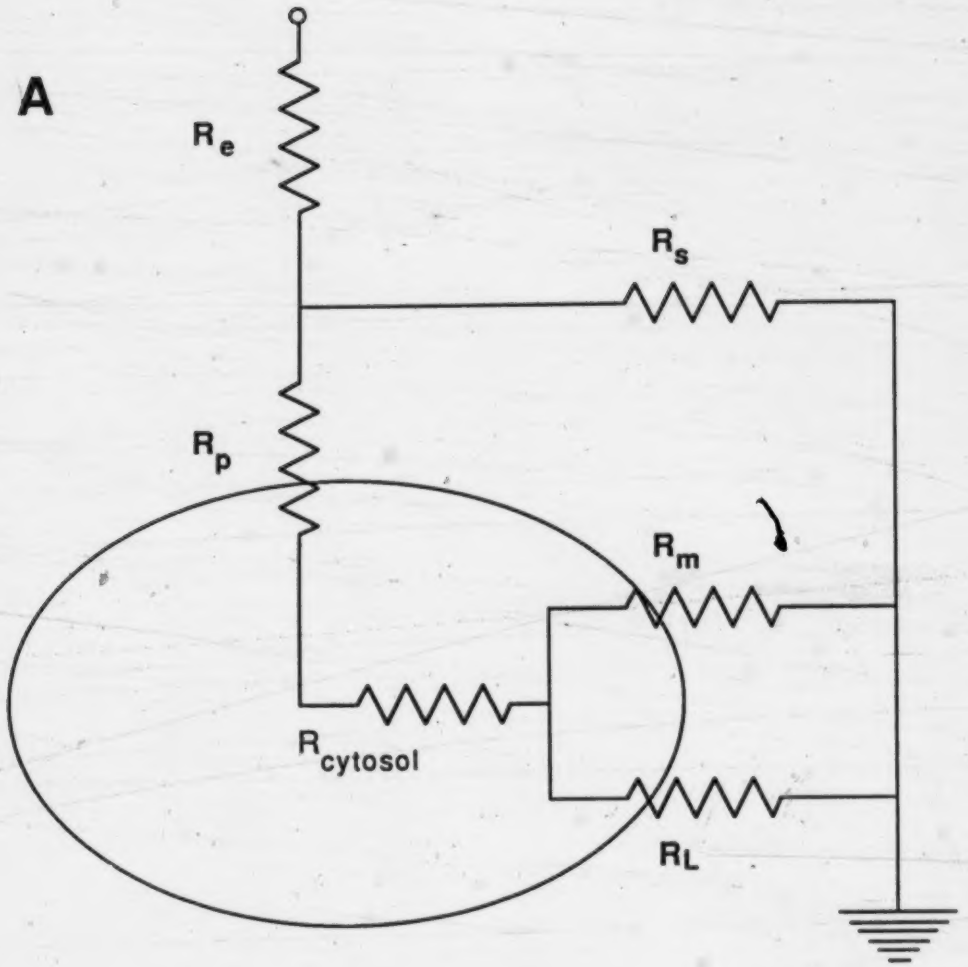
$$R_T = R_e + R_s, \text{ if } R_p >> R_m, R_L, R_s. \quad \text{-----} \quad (2)$$

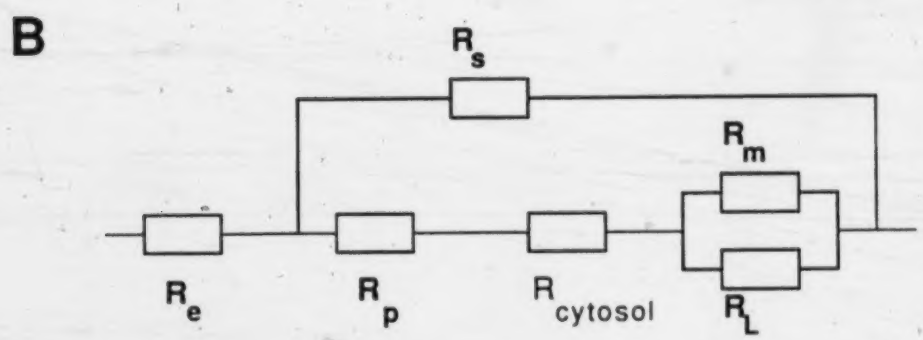
The seal resistance (R_s) can be measured using a voltage pulse, considering that R_s is much larger than R_e.

After rupture of the patch of membrane (that is to say, R_p is close to zero), the total resistance would be the following:

$$R_T = R_e + \{R_s \times R_x/(R_s + R_x)\} = R_s \times R_x/(R_s + R_x) \quad \text{-----} \quad (3)$$

If R_s were much greater than R_x (more than 10 times), the total resistance (R_T) which could be measured using a voltage pulse would represent R_x. This may not be the case in this study; R_s is unlikely to be much greater than R_x. Therefore, the resistance measured by repetitive hyperpolarizing voltage pulses of 5 to 10 mV would represent R_T in equation 3. Assuming that both R_m and R_L are constant at a given membrane potential (i.e. R_x is constant), a drastic change of R_T, either a decrease or increase, would then possibly represent a change of R_s. The total resistance is described in this study as the "leak" resistance to be distinguished itself from a leakage resistance (R_L), a membrane transverse resistance (R_m) or seal resistance (R_s).


A
R_e
R_s
R_p
R_m
$R_{cytosol}$
R_L


B
R_s
R_m
R_e
R_p
$R_{cytosol}$
R_L

Appendix 7.

Protocols for Histochemistry

A. Falck-Hillarp technique.

Monoamines condense with formaldehyde vapor to form 6,7-dihydroxy-3,4-dihydroisoquinolines (from catecholamines) or 6-hydroxy-3,4-dihydro-β-carbolines (from indolamines). These reaction products fluoresce and can be visualized under a UV microscope.

1) A desiccator was set up having 70% humidity (34.5 ml of 96% H_2SO_4 was added to 65.5 ml of distilled water).

2) Several beakers were filled with about 15 g each of fresh paraformaldehyde and placed in the desiccator. They were labelled with the date and allowed to equilibriate to 70% humidity for at least 10 days.

3) Freeze-drying techniques were not used as these tend to damage fragile tissue. Instead, stretch-drying procedures were used. The velum preparation (including ring canal) was pinned out on a Sylgard-coated microscope slide with cactus spines, washed several times with isotonic (0.694 M) sucrose solution to reduce the formation of salt crystals, and allowed to dry overnight in a desiccator over P_2O_5.

4) When the tissue had dried, it was placed in a container above a beaker containing paraformaldehyde. A wire platform inside a glass jar was used to support the slides and prevent them from touching the paraformaldehyde. The tightly-sealed jar was placed in an oven at 80 ^{o}C for 1 to 3 hours.

5) After heating, the cactus spines were removed from the preparation and it was mounted in desiccated liquid paraffin. It was then examined under a UV microscope.

Several controls were used :
1. Preparations were treated as outlined above but were not heated or heated in an empty, tightly sealed container.

2. To test specificity of fluorescence, preparations were immersed in a solution of sodium borohydride [1 to 2% (w/v)] in 90% alcohol for about 5 min. Alternatively, they were immersed under running tap water for 5 to 10 min. These procedures

reversibly reduce the fluorophores formed from catecholamines on condensation with formaldehyde vapor to their non-fluorescent 1,2,3,4-tetrahydro- derivatives. These quenched preparations can be reacted again with formaldehyde vapor and the fluorophores will be reformed.

References

Corrodi H, Jonsson G (1967) The formaldehyde fluorescence method for the histochemial demonstration of biogenic monoamines. A review on the methodology. *J. Histochem. Cytochem.* 15, 65-78.

Hamberger B, Malmfors T, Sachs C (1965) Standardization of paraformaldehyde and of certain procedures for the histochemical demonstration of catecholamines. *J. Histochem. Cytochem.* 13, 147.

B. Glyoxylic Acid method.

The modified glyoxylic acid (GA) method of de la Torre & Surgeon (1976) was followed with a small modification; HEPES buffer was used instead of phosphate buffer solutions for GA solutions.

For tissue preparations

1) A few drops of distilled water were placed on the surface of a precooled cryostat chuck and the tissue was oriented on it. The chuck was transferred immediately to the –30 °C cryostat.

2) Sections of different thicknesses (10, 20, 30 μm) were prepared when the tissue was frozen.

3) The sections were picked up with clean but nontreated room temperature glass slides by gently pressing the slide against the cryostat knife blade. Several seconds were allowed for the section to melt onto the slide.

4) The sections were dipped quickly in sucrose-HEPES-GA (SHG) solution at room temperature 3 times (1dip/sec) or in SHG solution at 0 °C for 10 min. Excess fluid was removed from the slide using Whatman filter paper.

5) The sections were dried with cool air and then baked at 80 °C in an oven for 1 to 3 min.

6) A few drops of mineral oil were placed near the tissue. It was coverslipped after being removed from the oven. The coverslipped slide was placed on a hot plate set at 80 °C for 90 sec. They were then examined under a UV microscope.

Control sections were treated in exactly the same way but were dipped in sucrose-HEPES solution (pH 7.4) instead of SHG solution. Difficulties were experienced since sections would not stick to the clean glass slides or they crumpled, causing severe tissue distortion. The use of gelatinized slides did not overcome this problem.

For cultured cells

1) Cultures were incubated at 12 °C in an incubator with precursors (0.1 mM of DOPA or tyrosine solutions) for 30 min and then washed twice with normal artificial sea water (NASW).

2) The above step was repeated.

3) The prepared cultures were washed twice with sucrose-HEPES solution and incubated at 4 °C with SHG solution for 1 to 5 min.

4) Cultures were air-dried at room temperature and then baked at 80 °C in an incubator for 90 sec. A drop of mineral oil was placed over the cultures and then the dish was coverslipped. They were then examined under a UV microscope.

Controls were not incubated in precursor solutions and/or SHG solutions but were otherwise treated the same way.

*SHG solution; 1% GA, 20 mM HEPES, and 0.6 M sucrose; pH was adjusted to 7.4 with 1N NaOH.

References

de la Torre JC, Surgeon JW (1976) A methodological approach to rapid and sensitive monoamine histofluorescence using a modified glyoxylic acid technique: the SPG method. *Histochem.* 49, 81-93.

C. Faglu-PEG method.

Biasi et al. (1984) obtained good resolution of morphological details and precise localization of neurons containing different monoamines in the pedal ganglia of *Mytilus*

using two modified Falck-Hillarp methods; aluminum formaldehyde (ALFA) method developed by Loren et al. (1982) and formaldehyde-glutaraldehyde polyethylene glycol (Faglu-PEG) technique by Scholer & Armstrong (1982).

1) Tissues were fixed in a solution of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 2h at 4 °C.

2) The fixed tissues were transferred to 30, 50, 70, 90% (V/V) solutions of molten PEG dissolved in fixative for 1 h each (The last passage in an oven at 40 °C).

3) The tissue blocks were infiltrated with 100% PEG at 40 °C for 2 h and then embedded in 100% molten PEG using small aluminum foil moulds. The moulds were left in a freezer for 5 min at -20 °C and then stored in a refrigerator to harden the wax rapidly and uniformly.

4) Sections (10 µm thick) were obtained with a sliding microtome and were floated on vials containing warmed fixative mixture. The sections were mounted on slides with paraffin oil and then examined under the UV microscope.

For controls, solutions without fixatives were used at step 2 (i.e., PEG without fixative) and step 4 (i.e., warmed buffered solutions without fixative).

References

Biasi D, Vitellaro-Zccarello L, Blum I (1984) Histochemical localization of monoamines and cholinesterase in *Mytilus* pedal ganglion. *Histochemistry* 81: 561-565.

Loren I, Bjorklund A, Lindvall O, Schmidt RH (1982) Improved catecholamine histofluorescence in the developing brain based on the magnesium and aluminum (ALFA) perfusion techniques: methodology and anatomical observations. *Brain Res. Bull.* 9: 11-26.

Scholer J, Armstrong WE (1982) Aqueous aldehyde (Faglu) histofluorescence for catecholamines in 2 nm sections using polyethylene glycol embedding. *Brain Res. Bull.* 9: 27-31.

Appendix 8.

Glyoxylic acid (GA) induced fluorescence in primary cultures of nerve-ring tissue of *Polyorchis penicillatus* which were pre-incubated with DOPA.

A. A control in which cells were not incubated with sucrose-HEPES-glyoyxlic acid (SHG) solution.

B. Some cells treated with DOPA for 30 min prior to processing with SHG exhibited a strong blue-white fluorescence. Triangles (Δ) denote cells producing specific fluorescence.

C. One of the fluorescent cells which were fluorescent is shown at higher magnification. The cell had a thin and long process (Δ). [n.b. another cell (■) seen in the panel did not show specific fluorecence.]

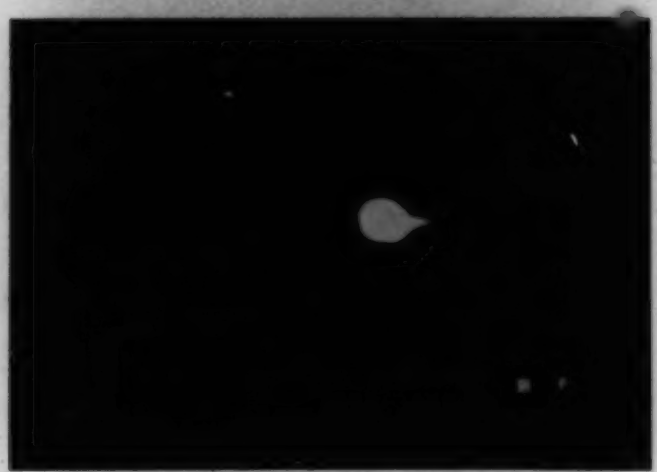
A.

100μm

B.

100μm

C.

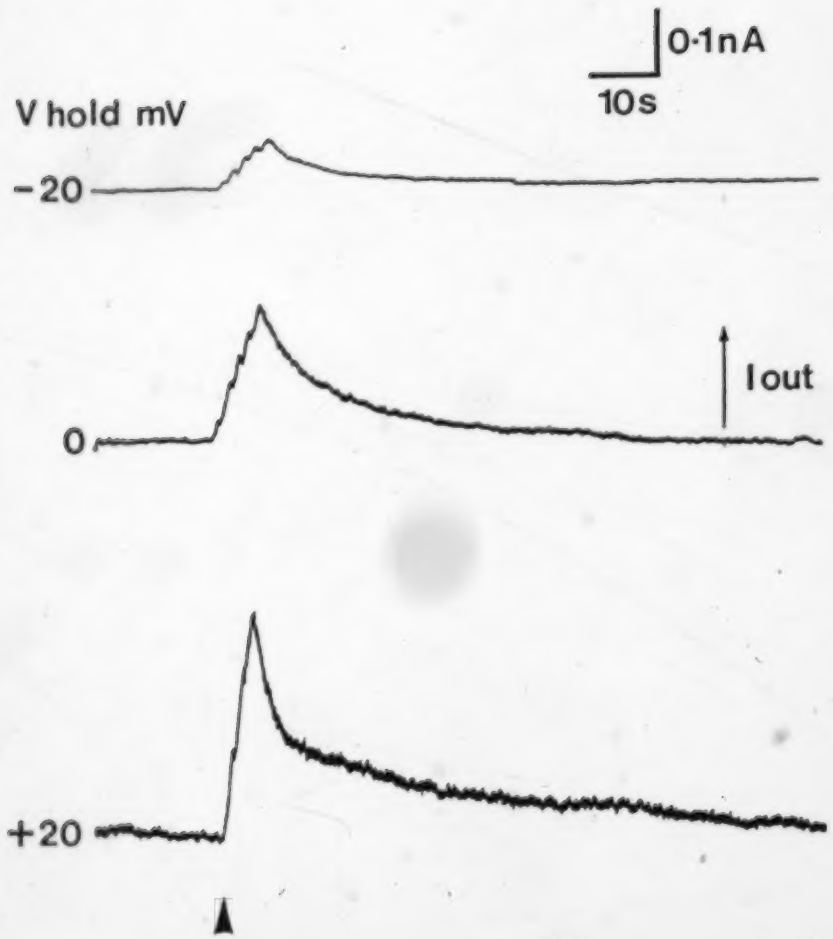


50μm

Appendix 9

Responses of an isolated swimming motor neuron to applications of Poly-A-peptide. Each application at a different holding potential consisted of five consecutive ejections of Poly-A peptide at 10^{-6} M for 999 ms each at 138 KPa. [n.b. Both activation and inactivation kinetics of peptide responses were significantly different from those of dopamine responses.]

V clamp of SMN



5 x 1s pulses 10^{-6} M Poly A